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Pharmacological Characterization of Presynaptic Voltage-Dependent Calcium Channels

by

Mary Margaret Thomas

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Department of Pharmacology

Edmonton , Alberta

Spring 1996

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Pharmacological Characterization of Presynaptic Voltage-Dependent Calcium Channels submitted by Mary Margaret Thomas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Marek Michalak

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Christina Benishin

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27th December, 1995

To my Mom and Dad

Abstract

Presynaptic calcium channel pharmacology was studied using synaptosomal preparations from rat cerebral cortex and stopped-flow fluorimetry. Synaptosomes were loaded with the fluorescent Ca^{2+} chelating dye, Fura-2, by incubation with the membrane permeant acetoxymethyl ester derivative. Depolarization by elevating the external K^+ concentration or by blocking potassium channels with 4-aminopyridine (4-AP) caused a rapid increase in cytoplasmic Ca^{2+} as measured by a quench in Fura-2 fluorescence when excited at 390 nm. The fluorescence change could be reasonably fit by a single exponential process and the magnitude of the response was voltage-dependent. The quench was blocked by micromolar concentrations of Cd^{2+}, Co^{2+} and La^{3+}. 1,4-Dihydropyridines (L-type ligands) did not generally affect the Ca^{2+} signal. ω-Conotoxin GVIA (N-type ligand), at nanomolar toxin concentrations, blocked 10 to 15% of the K^+-induced signal and 25% of the 4-AP-induced signal. Regardless of the depolarization method employed, ω-agatoxin IVA and the polyamine FTX from the spider, *Agelenopsis aperta* (P-type ligands), or ω-contoxin MVIIC (Q/P-type ligand) from the fish-hunting snail *Conus magus* blocked 25-40% of the response. 2-Chloroadenosine inhibited 10-15% of the Ca^{2+} signal, while other drugs postulated to modulate calcium channels were without effect in the current system. IgG, purified from individuals diagnosed with amyotrophic lateral sclerosis did not alter the presynaptic Ca^{2+} response in any systematic way. The results demonstrate the sensitivity of stopped-flow fluorimetry for measurements of rapid Ca^{2+} signals in mammalian presynaptic terminals and indicate that voltage-dependent Ca^{2+} uptake by mammalian nerve terminals is mediated primarily by calcium channel subtypes sensitive to P- and Q-subtype ligands.

In order to correlate the results of the calcium channel studies with the functional response of neurotransmitter release, a number of release assays were evaluated. None of the techniques investigated provided both rapid time resolution and adequate sensitivity for measuring transmitter release from synaptosomes. Additional work is required to confirm that the Ca^{2+} influx through calcium channels, characterized pharmacologically in this study, is critical for triggering neurotransmitter release.

Acknowledgement

So here it is....a thesis; the result of several years of study at the University of Alberta, Department of Pharmacology....although not a small thing, I would hope and pray that it is not the only consequence of my sojourn here in Edmonton! Nevertheless the completion of this work has been a primary focus of my energies for the last five years and certainly would not be finished without the support of many people, not least of which is my supervisor, Dr. Susan Dunn. I appreciate the fact that Susan has been a consistent and solid advocate on my behalf throughout my program. Susan provided considerable resources towards developing an interesting and challenging research experience for me and I am grateful for her supervision. I am also much obliged to Dr. Bill Colmers and Dr. Marek Michalak who served on my supervisory committee and provided generally thoughtful, sometimes obscure, and occasionally, truly remarkable tutelage throughout the course of my studies. Dr. Christina Benishin and Dr. Bill Dryden have also contributed in various ways... thank you.

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"Of the making of many books there is no end and much study wearies the body....but here is the conclusion of the matter: Fear God and keep his commandments, for this is the whole duty of man"

Ecclesiastes 12:12-13.

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List of symbols/nomenclature/abbreviations

4-AP	4-aminopyridine
ACh	acetylcholine
ALS	amyotrophic lateral sclerosis
AMP	adenosine monophosphate
ANOVA	analysis of variance
AOAA	aminooxyacetic acid
ATP	adenosine triphosphate
$[Ca^{2+}]_i$	intracellular/ intrasynaptosomal Ca^{2+} concentration
CNS	central nervous system
dH_2O	distilled, filtered water
$diSC_3(5)$	3,3'-dipropylthiodicarbocyanine
EGTA	ethyleneglycol tetraacetic acid
FTX	polyamine toxin from the Funnel Web spider *Agelenopsis aperta*
GABA	γ-aminobutyric acid
g_{max}	maximum gravitional force
$h\nu$	photon
Hepes-Na^+	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, Na^+ salt
HPLC	high performance liquid chromatography
IC_{50}	drug concentration giving 50% maximal inbibition
IgG	immunoglobulin G
$NADP^+$	nicotinamide adenine dinucleotide phosphate

NPY	neuropeptide Y
OPA	o-phthaldialdehyde
PBS	physiological buffered saline
RMD	rapid mixing device
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEM	standard error of the mean
sFTX	synthetic FTX
Tris	tris(hydroxymethyl)aminomethane
TTX	tetrodotoxin

Chapter One
General Introduction

All cells share many common metabolic processes; however different cell types are specialized for particular functions. Neurons are specifically designed to receive, process and transmit information. To understand the ways by which these different neuronal functions are accomplished is the fundamental basis of neuroscience.

Developments beginning a few hundred years ago set the groundwork for subsequent investigations of the nervous system. Initial focus was on the relationship of the brain to the periphery, particularly with respect to muscle control. Nerve cells were first visualized by microscopy in the 19th century and the meticulous work by Ramon y Cajal, Golgi and others began to reveal the variety of cells present in neural tissues (reviewed by Brazier, 1988). Although nerve cells could be identified over one hundred years ago, knowledge of the ways in which they were involved in neural transmission accumulated only gradually.

Sherrington was the first to use the term synapse to designate the site of transfer of information from one nerve cell to the next (Sherrington, 1906). Although there was little argument that a signal was being transmitted from cell to cell, there was much debate as to whether the signal was chemical or electrical in nature (Dale, 1937; Eccles, 1946; 1975). The electrical excitability of nerve cells had been established for several decades before Hodgkin, Huxley and co-workers demonstrated that differential permeability of the membrane to potassium and sodium ions formed the basis of the observed electrical properties (Hodgkin & Huxley, 1952; Young, 1975). Voltage sensitive ion channel proteins located in the plasma membrane were subsequently shown to account for these distinctive properties of neurons (reviewed by Hille, 1992). The main point of contention in the controversy over whether the signal was chemical or electrical was the necessity to account for the rapid speed of transmission in the central nervous system. It was difficult to imagine how a chemical signal would be able to act so quickly (Eccles, 1975). However support for a chemical basis of transmission included the relatively prolonged membrane potential changes, the unexpected direction

of current flow measured in post-synaptic cells following an inhibitory signal and data from numerous studies demonstrating post-synaptic effects of application of specific chemical solutions (Dale, 1937; Nachmansohn, 1946; Eccles, 1975). Eventually chemical neurotransmission became an established fact of neuroscience.

Release of neurotransmitter occurs from specialized regions at nerve endings as well as at varicosities throughout the dendritic tree of the neuron. These particular sites form the presynaptic region of the synapse and are in close proximity to the membranes of other, post-synaptic cells. In these presynaptic regions or terminals, there are many distinct structures or molecular entities that have been shown to have a role in mediating transmitter release. Synaptic vesicles are one structural component evident in electron microscopy or freeze-fracture studies (Gotow, 1992). There are a number of different types of synaptic vesicles, apparently specific for particular chemical transmitters (Verhage *et.al.*, 1994; Sihra & Nichols, 1993; Régnier-Vigouroux & Huttner, 1993). The vesicles have been shown to contain high affinity protein carriers for the uptake of specific neurotransmitters, in addition to enzymes for the synthesis of neurotransmitters from precursors. They also contain membrane-associated proteins that bind Ca^{2+} with high affinity and have been shown to release their contents out of the cell by exocytosis (Kish & Ueda, 1989; Régnier-Vigouroux & Huttner, 1993; Jahn & Südhof, 1994; Bennett & Scheller, 1994; Fried, 1995).

The process of exocytosis is Ca^{2+}-dependent and requires a complex interaction of vesicle-associated proteins with other specific proteins found in the plasma membrane and cytosol (Verhage *et.al.*, 1994; Jahn & Südhof, 1994; Bennett & Scheller, 1994). There is limited evidence for internal Ca^{2+} stores in nerve terminals (Gotow, 1992; Zimmermann, 1990; Tareilus *et.al.*, 1993; Verhage *et.al.*, 1994) and the speed with which transmitter is released following the arrival of an action potential at the terminal (< 1 msec), precludes a role for Ca^{2+} release from internal stores in triggering the initial transmitter release.

Katz and Miledi first demonstrated that Ca^{2+} entry is a necessary requirement for neurotransmission (Katz & Miledi, 1968). In their studies, and many electrophysiological studies that followed, removal of Ca^{2+} from the extracellular bathing medium resulted

in a loss of transmission. Under physiological resting conditions, the $[Ca^{2+}]_i$ is about 10-100 nM, while the external concentration is 1-2 mM (Hodgkin & Keynes, 1957). There is thus a significant Ca^{2+} concentration gradient across the membrane. Depolarization of the nerve terminal activates voltage-dependent calcium channels and the electrochemical gradient favours rapid influx of Ca^{2+} through any open channel. Models of the mechanism of Ca^{2+} action in neurotransmitter release suggest that a relatively low affinity Ca^{2+}-binding site(s) exists very close to the inner side of the calcium channel pore (Almers, 1994; Bennett & Scheller, 1994). Upon the binding of Ca^{2+} to this site, the process of synaptic vesicle exocytosis is triggered. Since the response induced by the entry of Ca^{2+} into the presynaptic terminal occurs very rapidly, it is thought that some synaptic vesicles are positioned for immediate release in an "active zone" which includes the voltage-dependent calcium channels (Verhage *et.al.*, 1994; Jahn & Südhof, 1994). Due to the time constraints of release and the high Ca^{2+} buffering capacity of the intracellular fluid, the Ca^{2+} that enters through the channel cannot diffuse far into the cytoplasm. Thus the site(s) of action must be very close to the cytoplasmic side of the channel protein (Augustine *et.al.*, 1991; Almers, 1994; Verhage *et.al.*, 1994). Recently there has been considerable progress in identifying some of the proteins involved in the initiation or regulation of transmitter release (Jahn & Südhof, 1994; Bennett & Scheller, 1994; Fried, 1995). Several proteins, including synaptobrevin and syntaxin, which are specifically associated with the synaptic vesicles and plasma membrane respectively, are proposed to form a "docking complex" by which synaptic vesicles are positioned for rapid exocytotic release (Jahn & Südhof, 1994; Bennett & Scheller, 1994). Other proteins such as synaptophysin and synaptotagmin are thought to be involved in the "calcium trigger" for release because these proteins have specific binding sites for Ca^{2+} and, in the presence of micromolar concentrations of Ca^{2+}, have been shown to complex with phospholipids (Südhof *et.al.*, 1993; Bennett & Scheller, 1994). Many of these proteins are also regulated by phosphorylation and most have been shown to be critical for normal neurotransmitter release (Greengard *et.al.*, 1993; Fried, 1995).

Voltage-Dependent Calcium Channels: As noted above, the Ca^{2+} signal for transmitter release arises from entry of Ca^{2+} through voltage-dependent calcium channels located in the active zone of the presynaptic terminal. Voltage-dependent calcium channels are ubiquitous in the membranes of excitable cells where they mediate the rapid Ca^{2+} influxes involved in the regulation of many cellular functions including muscle contraction and neurotransmitter release (Miller & Fox, 1990) In neurons and other tissues five main subtypes of voltage-dependent calcium channels, termed L, T, N, P and Q, have been defined on the basis of differences in their electrophysiological and pharmacological profiles and additional subtypes are postulated to exist (Carbone & Swandulla, 1989; Miller & Fox, 1990; Bertolino & Llinás, 1992; Miljanich & Ramachandran, 1995). This subtype classification is an operational one which, for convenience, is used throughout this thesis. However, since it is based on differences in functional properties and not yet on detailed knowledge of the different structures of channel subtypes, it is likely that there is some overlap between the different classes and that some channels may display characteristics common to more than one class.

The best characterized channels are the L-type which are sensitive to a variety of calcium channel ligands, including 1,4-dihydropyridine derivatives. The other subtypes of calcium channels are insensitive to dihydropyridines, but N-type channels are blocked by a peptide toxin from *Conus geographus*, ω-conotoxin GVIA (Carbone & Swandulla, 1989; Miljanich & Ramachandran, 1995). T-Type channels are distinguishable by their low voltage of activation (-50 to -70 mV) but study of these channels has been difficult since their pharmacological properties have not been well developed (Miller & Fox, 1990). The P-type calcium channel was first identified in cerebellar Purkinje cells and has since been localized to several regions of the nervous system (Llinás *et.al.*, 1992). P-type currents are potently blocked by a low molecular weight polyamine fraction (FTX) from the venom of the funnel web spider, *Agelenopsis aperta* (Cherksey *et.al.*, 1991; Llinás *et.al.*, 1989). Several peptide toxins (ω-agatoxins) have also been isolated from funnel web spider venom and these have been shown to block Ca^{2+} currents in some tissues that are otherwise insensitive to L- and N-type calcium channel antagonists (Mintz

et.al., 1992; Venema *et.al.*, 1992). A fifth channel subtype, termed the Q-channel has been proposed and is defined by a relatively high sensitivity to ω-conotoxin MVIIC (Olivera *et.al.*, 1994). The P-type channel is also sensitive to ω-conotoxin MVIIC, but at somewhat higher concentrations (Olivera *et.al.*, 1994; Wheeler *et.al.*, 1994). Most functional studies of calcium channels have indicated that Ca^{2+} influx and transmitter release in the mammalian central nervous system is significantly blocked only by antagonists of N, P and Q-type channels (this thesis; Hillyard *et.al*, 1992; Mintz *et.al.*, 1992; Turner *et.al.*, 1992; 1993; Uchitel *et.al.*, 1992; Zhang *et.al.*, 1993; Thomas et.al., 1994; Wheeler *et.al.*, 1994). Table 1.1 summarizes the pharmacological antagonists and some of the identified functional roles of the different calcium channel subtypes in neurons.

Table 1.1

Pharmacological Antagonists and Functional Significance of Subtypes of Voltage-Dependent Calcium Channels

Subtype	ANTAGONISTS	PROPOSED FUNCTIONS
L	1,4 dihydropyridines, benzothiazepines, phenylakylamines	signal transduction/ action potential generation
N	ω-conotoxin GVIA (from *Conus geographus*)	neurotransmitter release
T	amiloride	neuronal oscillatory activity
P	ω-agatoxin IVA; FTX; ω-agatoxin IVB (from *Agelenopsis aperta*)	neurotransmitter release
Q	ω-conotoxin MVIIC (from *Conus magus*)	neurotransmitter release
O\R	none of the above	?

Molecular Biology

L-type or dihydropyridine sensitive calcium channels are found in relative abundance in the transverse-tubule membranes of skeletal muscle. This source of channel protein has permitted its purification and the characterization of its subunit structure. The L-type channel protein has five subunits, named α_1, α_2, β, γ, and δ (reviewed by Catterall, 1995). The α_2 and δ subunits are encoded by the same gene and appear to be disulfide-linked. Expression of the α_1 subunit alone in cultured cell lines results in a functional channel, sensitive to voltage and pharmacological agents (reviewed by Hofmann *et.al.*, 1994; Catterall, 1995). The other subunits appear to play a modulatory role since co-expression of additional subunits with α_1 have been shown to modify various aspects of the function of the expressed channel (Singer *et.al.*, 1991; Isom *et.al.*, 1994).

The kinetics of activation and inactivation of the calcium channel are complex but current data suggest significant roles for the auxiliary subunits in regulation of these activities (Isom *et.al.*, 1994; McCleskey, 1994; Catterall, 1995). β subunits, in particular, appear to have a role in facilitating channel opening (Catterall, 1995). Expressed β subunits also contain several consensus sequences for phosphorylation and have been shown to be specifically dephosphorylated by phosphoprotein phosphatases (Lai *et.al.*, 1993). Thus the phosphorylation state of the β subunit may be one of the molecular means for regulation of channel activity (Isom *et.al.*, 1994)

The primary sequence of the α_1 subunit contains four similar domains, each having six putative membrane spanning regions termed S1-S6. Each domain is homologous to the *Shaker* potassium channel (Jan & Jan, 1989) and the calcium channel sequence also shares a high degree of homology with the previously purified and cloned sodium channel α subunit (reviewed by Catterall, 1995). Molecular biological approaches have helped to identify specific domains or amino acids in the primary sequence that are important in determining the selectivity and permeability characteristics of these ion channels. Several amino acids in the region linking S5 and S6 have been shown to be important in regulating channel conductance, suggesting that this region

forms at least part of the pore-lining structure in the channel (Hofmann *et.al.*, 1994; Catterall, 1995). The voltage sensitivity of the channel proteins appears to arise from regularly spaced positive charges in the S4 region of each domain of the calcium and sodium channels or the homologous S4 region of the potassium channel (Catterall, 1995). These charges are postulated to shift slightly relative to negative charges in the protein or membrane when the electric field changes with depolarization. These charges have therefore been called gating charges (Jan & Jan, 1989; Catterall, 1995) and it is movement of these charges that is thought to induce the protein conformational change that opens the channel.

The cDNA of the α_1 subunit of the skeletal muscle L-type calcium channel (α_{1S}) has been used to probe other tissues for the presence of voltage-dependent calcium channels (Catterall, 1995). This approach has led to the identification and cloning of several α_1 subtypes. In addition to α_{1S}, five classes of α_1 subunits with the nomenclature A-E have been described (Zhang *et.al*, 1993: Birnbaumer *et.al.*, 1994; Hofmann *et.al.*, 1994). Two of these classes, α_{1C} and α_{1D}, form channels that are sensitive to modulation by dihydropyridines and these are found primarily in cardiac and neuroendocrine tissues, but they have also been identified in neurons and other cell types. The remaining three classes have been found only in neuronal tissue (Olivera *et.al.*, 1994). α_{1B} has some sensitivity to ω- conotoxin GVIA , suggesting that it may be a component of the N-type channel, while ω-agatoxin IVA , which is selective for the P-type channel, apparently blocks α_{1A} with high potency. ω-Conotoxin MVIIC interacts with both α_{1A} and α_{1B} subunits (Miljanich & Ramachandran, 1995). The demonstration that specific α_1 subunits correlate quite well with specific pharmacological agents for the different channel subtypes, provides some basis for anticipating distinct functions of these channels.

Regulation of Calcium Channels by Second Messenger Systems: The activity of voltage sensitive calcium channels may be modulated by a variety of receptor/G-protein/effector systems (Dolphin, 1990; Scott *et.al.*, 1991; Fox *et.al.*, 1991; Brown, 1993). A well characterized example is the G-protein linked activation of adenylate

cyclase following β-adrenergic stimulation in cardiac tissue. Elevated cyclic AMP levels result in cyclic AMP-dependent phosphorylation of cardiac L-type calcium channels and increased flux of Ca^{2+} (reviewed by Fox *et.al.*, 1991). In sensory neurons, cyclic AMP, phospholipase C activation and other soluble second messenger systems acting on calcium channels have been reported in studies using whole cell recording techniques (Dolphin, 1990; Scott *et.al.*, 1991; Brown, 1993). In most cases, the consequence is an inhibition of the neuronal Ca^{2+} current (Brown, 1993). Voltage-sensitive calcium channels have also been shown to be modulated by a direct interaction of the G-protein with the channel protein in some tissues (Scott *et.al.*, 1991; Brown, 1993). A possible feedback mechanism that could adjust presynaptic function would be served if similar agonist-induced effects occur at the synaptic terminal. Direct studies of the second messenger effects on presynaptic Ca^{2+} currents are complicated due to the small size of the presynaptic terminal; however modulation of presynaptic calcium channel activity by second messenger systems provides many pathways for possible functional regulation of neurotransmission.

Role of Voltage-Dependent Calcium Channels in Neurotransmitter Release: As already noted, exocytotic release of neurotransmitter requires the entry of Ca^{2+} into the presynaptic terminal. Electrophysiological recordings from dissociated or cultured neuronal cells have demonstrated the existence of Ca^{2+} currents that may be distinguished on the basis of their sensitivity to various calcium channel antagonists (Regan *et.al.*, 1991; Zhang *et.al.*, 1993; Wheeler *et.al.*, 1994). Peptide toxins from *Conus geographus* and *Conus magus*, two species of fish-hunting snails, as well as agatoxins from the funnel web spider, *Agelenopsis aperta*, have been shown to be the most efficacious antagonists in studies of neurotransmitter release (Olivera *et.al.*, 1994).

Although the presynaptic terminal of mammalian central synapses is too small for direct recordings, measurements of post-synaptic cell responses reflect the events of synaptic transmission, including events occurring presynaptically. Slice preparations which permit recording from both presynaptic and postsynaptic neurons, have been

effectively used to investigate the functional role of different calcium channel subtypes in transmission. Results of studies with the rat hippocampal slice preparation indicate that synaptic transmission between the CA3 and CA1 regions is reduced in the presence of ω-conotoxin GVIA, suggesting that N-type calcium channels are involved in mediating transmission at this synapse (Wheeler *et.al.*, 1994). A recent report, in which additional peptide toxins were investigated, demonstrated that ω-agatoxin IVA blocked about 25% of the recorded presynaptic Ca^{2+} transients, while ω-conotoxin GVIA blocked almost 40% of the signal. Both ω-agatoxin IVA and ω-conotoxin GVIA-sensitive transients, as well as some additional current, were blocked by the application of ω-conotoxin MVIIC (Wu & Saggau, 1995). Neurotransmitter release studies using isolated presynaptic terminals prepared from mammalian central nervous system tissues have also indicated the presence of multiple subtypes of calcium channels at the presynaptic terminal (Pocock & Nicholls, 1992; Turner *et.al.*, 1993; Carvalho *et.al.*, 1995; Turner *et.al.*, 1995).

Role of Voltage-Dependent Calcium Channels in Disease: Due to the importance of calcium channels in regulating neurotransmission and other cellular functions, it is not surprising to find alterations of channel function in several disease processes. Therapeutically, antagonists of the L-type calcium channel have been used for many years (Triggle & Janis, 1987). 1,4-Dihydropyridines are used to treat congestive heart failure and hypertension because of the vasodilatory effects of these drugs (Uski *et.al.*, 1994). In cardiac tissue, the calcium channel antagonists, verapamil and diltiazem have been clinically useful in treatment of arrhythmias and angina pectoris (Uski *et.al.*, 1994; Triggle, 1994). Therapeutic manipulations in other tissues are not yet well developed; however antagonism of voltage-dependent calcium channels in the central nervous system may have potential for protection from ischemic damage and age-related neural degeneration (Scriabine *et.al*, 1989; Miljanich & Ramachandran, 1995). SNX-111 is synthetic ω-conotoxin-MVIIA, another peptide toxin from *Conus magus*, and is the first N-type calcium channel antagonist to be used in clinical studies as a neuroprotective agent (Miljanich & Ramachandran, 1995).

A disruption of calcium homeostasis, possibly due to dysfunction of Ca^{2+} uptake mechanisms in neurons, has long been postulated as a factor in the progression of neurodegenerative processes such as Huntington's, Alzheimer's, and Parkinson's diseases (Sen *et.al.*, 1994; Khachaturian, 1994). Recently links between voltage dependent calcium channels and certain autoimmune based disorders have been established. In patients with Lambert-Eaton Myasthenic Syndrome (LEMS), a disease in which neuromuscular transmission is impaired, immunoglobulins directed against presynaptic calcium channels have been identified (Hewett& Atchison, 1992; Seagar *et.al.*, 1993). The antibodies decrease Ca^{2+} influx, thereby decreasing synaptic efficiency. It is not clear if a single subtype of channel is affected or if the immunoglobins interact more generally with all types of presynaptic calcium channels. There is some evidence that N, P or Q sub-types are primarily involved (Vincent *et. al.*, 1994; Grassi *et.al.*, 1994; Miljanich & Ramachandran, 1995).

Recent reports by several groups have provided some indication that autoimmunity to voltage-sensitive calcium channels may account for some aspects of another motorneuron disease, amyotrophic lateral sclerosis (ALS) (Appel *et.al.*, 1993; Llinás *et.al.*, 1993; Kimura *et.al.*, 1994; Smith *et.al.*, 1994; Zhainazorov *et.al.*, 1994). Among the first observations that suggested a possible presynaptic-directed, immune-mediated factor in ALS, was an increase in minature end plate potential frequency at the mouse neuromuscular junction in the presence of ALS IgG (Appel et.al., 1991). Evidence that the immunological effect was on calcium channels included the demonstration of specific interactions of purified patient IgG with the α_1 subunit of the L-type channel (Kimura *et.al.*,1994). In addition, Ca^{2+} currents through dihydropyridine sensitive channels of skeletal t-tubule membranes, whether in intact vesicles or purified and reconstituted in artificial bilayers, were reduced in the presence of ALS IgG (Delbono *et.al.*, 1991). In dissociated, cerebellar Purkinje cells, Ca^{2+} currents sensitive to P-type antagonists, were observed to increase in the presence of ALS IgG (Llinás *et.al.*, 1993) while in another study, in which serum fractions rather than purified IgG were applied to cerebellar granule cells, there was a decrease in high threshold Ca^{2+} current (Zhainazarov *et.al.*,

1994). ALS IgG was found to be cytotoxic to a motoneuron cell line; however the cytotoxicity was reduced in the presence of ω-conotoxin GVIA and ω-agatoxin IVA, suggesting the ALS IgG effect was being mediated through the N or P-type calcium channel (Smith *et.al.*,1994). Thus, although different specific effects of IgG on calcium channel function have been reported in these studies, and the link between altered calcium channel function and the pathophysiology of the disease remain obscure, there is a reasonable pool of experimental evidence that an autoimmune mechanism involving voltage dependent calcium channels may be a factor in this disease.

The Synaptosome as a Model System

A number of experimental systems have been employed in studying synaptic transmission. Invertebrate preparations such as *Aplysia*, squid giant synapse, crayfish neuromuscular junction and frog neuromuscular junction, have been successfully used to establish basic principles and use of these preparations continues to contribute to our understanding of presynaptic mechanisms (Zimmermann, 1990; Augustine *et.al.*, 1991). Advances in electrophysiological techniques have permitted recordings to be made in tissue slice preparations and from acutely dissociated neurons or cultured cells, the latter being useful in functional expression studies of specific proteins postulated to be important in different aspects of the transmitter release process (Elferink *et.al.*, 1993; Geppert *et.al.*,1994). Isolated nerve terminals (synaptosomes) are another widely used preparation for biochemical and metabolic studies. Synaptosomes are membrane-bound vesicles that may be prepared from invertebrate and vertebrate, peripheral and central nervous system synaptic tissues (Whittaker, 1984).

Whittaker and co-workers were the first to isolate synaptosomes some thirty years ago (reviewed by Whittaker, 1993). There are several potential advantages in working with this subcellular fraction, including the relatively high purity achievable, the maintained metabolic activity of the preparation, the integrity of the intracellular components and the versatility of the preparation (Whittaker, 1984; Gordon-Weeks, 1991; Nicholls, 1993; Sihra & Nichols, 1993). Many groups have used this preparation

for investigating basic metabolic and cellular processes in neural tissues (Erecińska *et.al.*, 1994; Kauppinen, 1994). However, because synaptosomes contain all the necessary components for uptake, storage and release of neurotransmitters, they have been more widely used as a model for studies of presynaptic mechanisms in neurotransmission, including Ca^{2+} influx, neurotransmitter release and modulation of release by various second messenger systems (McMahon & Nicholls, 1991).

The nature of the synaptosomepreparation requires that depolarization be achieved by means other than an action potential. A number of chemical techniques for depolarizing the synaptosome membrane have been used (Bernath, 1992; Nicholls, 1993). The most common method is to increase the K^+ concentration of the bathing solution. Other chemical techniques include activation of sodium channels by veratridine or decreasing K^+ permeability with potassium channel blockers such as dentrotoxin or aminopyridines (Chapell *et.al.*, 1992; Herrero *et.al.*, 1991; Hu, 1993; Tibbs *et.al.*, 1989b).

Polarized neural plasma membranes are quite permeable to K^+ relative to other ions. The membrane potential, is described by the Nernst equation

$$\begin{aligned} E_m &= RT/zF \ln([ion]_o/[ion]_i) \\ &\approx RT/zF \ln([K^+]_o/[K^+]_i) \end{aligned}$$

where R is the gas constant; T is the absolute temperature; F is the Faraday constant; z is the valence and $[x]_o$ and $[x]_i$ are the extracellular and intracellular concentrations of the ion respectively. (Hille, 1992). Since E_m approximates the equilibrium potential for K^+, elevation of the external K^+ concentration results in a reduced potential difference across the membrane. In synaptosomes, increased K^+ causes a one-time clamped depolarization that is insensitive to tetrodotoxin (TTX) (Deutsch & Rafalowska, 1979; Tibbs *et.al.*, 1989b), a toxin from puffer fish that specifically blocks voltage-sensitive sodium channels (Catterall, 1995). This indicates that the depolarization observed is not dependent on Na^+ influx (Nicholls, 1993). Figure 1.1 schematically illustrates the effect of high K^+ depolarization on synaptosomes.

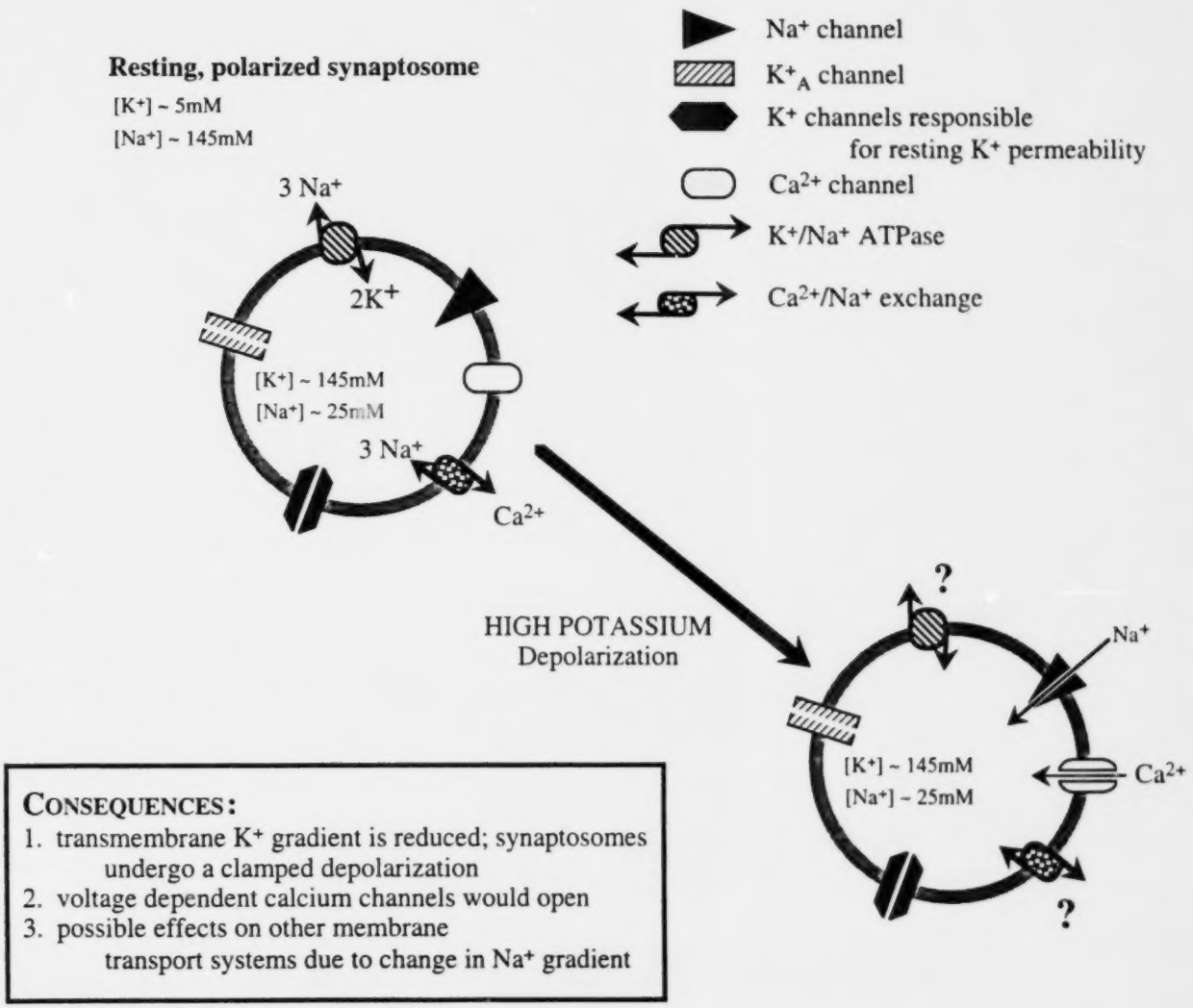


Figure 1.1 A schematic diagram of the consequences in synaptosomes of high K^+ depolarization. Elevating the external $[K^+]$ depolarizes the membrane according to the parameters of the Nernst equation. The primary change effected is a reduction in the K^+ gradient across the membrane leading to a one-time depolarization.

Depolarization may also be achieved by preventing the inactivation of sodium channels by a toxin such as veratridine, thus permitting an influx of Na^+ down its concentration gradient (Nicholls, 1993). The possible effects of this chemical depolarization are illustrated in Figure 1.2.

Depolarization by veratridine is blocked in the presence of TTX. The permanent depolarization resulting from this influx of Na^+ results in greater energy demands on the synaptosome vesicles due to stimulation of Na^+-K^+ ATPase. More calcium-independent release of transmitter is often reported with veratridine stimulation, probably due to the high internal [Na^+] which leads to reversed operation of the Na^+-coupled transmitter uptake carriers and subsequent increase in $[Ca^{2+}]_i$ by this route (McMahon & Nicholls, 1991; Bernath, 1992; Nicholls, 1993).

Recently, Nicholls and co-workers have argued that depolarization of mammalian cortical synaptosomes with 4-aminopyridine (4-AP) is more "physiological" than either high K^+ or veratridine stimulation (Tibbs *et.al.*, 1989b, Nicholls, 1993). 4-AP blocks at least two components of presynaptic K^+ conductance including a transient and delayed-rectifier-like current (Bartschat & Blaustein, 1985); however the transient current was shown to be the most sensitive to 4-AP. Block of the transiently activating K^+ channels results in increased membrane excitability leading to "action potentials" as Na^+ channels activate and then inactivate. The membrane is repolarized by K^+ influx through additional potassium channels postulated to be similiar to delayed rectifier potassium channels identified in neuronal cell bodies. It has been suggested that the synaptosome is able to recover, at least partially, from this depolarization since the ionic gradients across the membrane are not so radically altered as when high K^+ or veratridine stimulation is employed (Tibbs *et.ai.*, 1989a; 1989b; Nicholls, 1993; Nichols *et.al.*, 1994). Figure 1.3 shows a schematic diagram of changes induced by 4-AP depolarization.

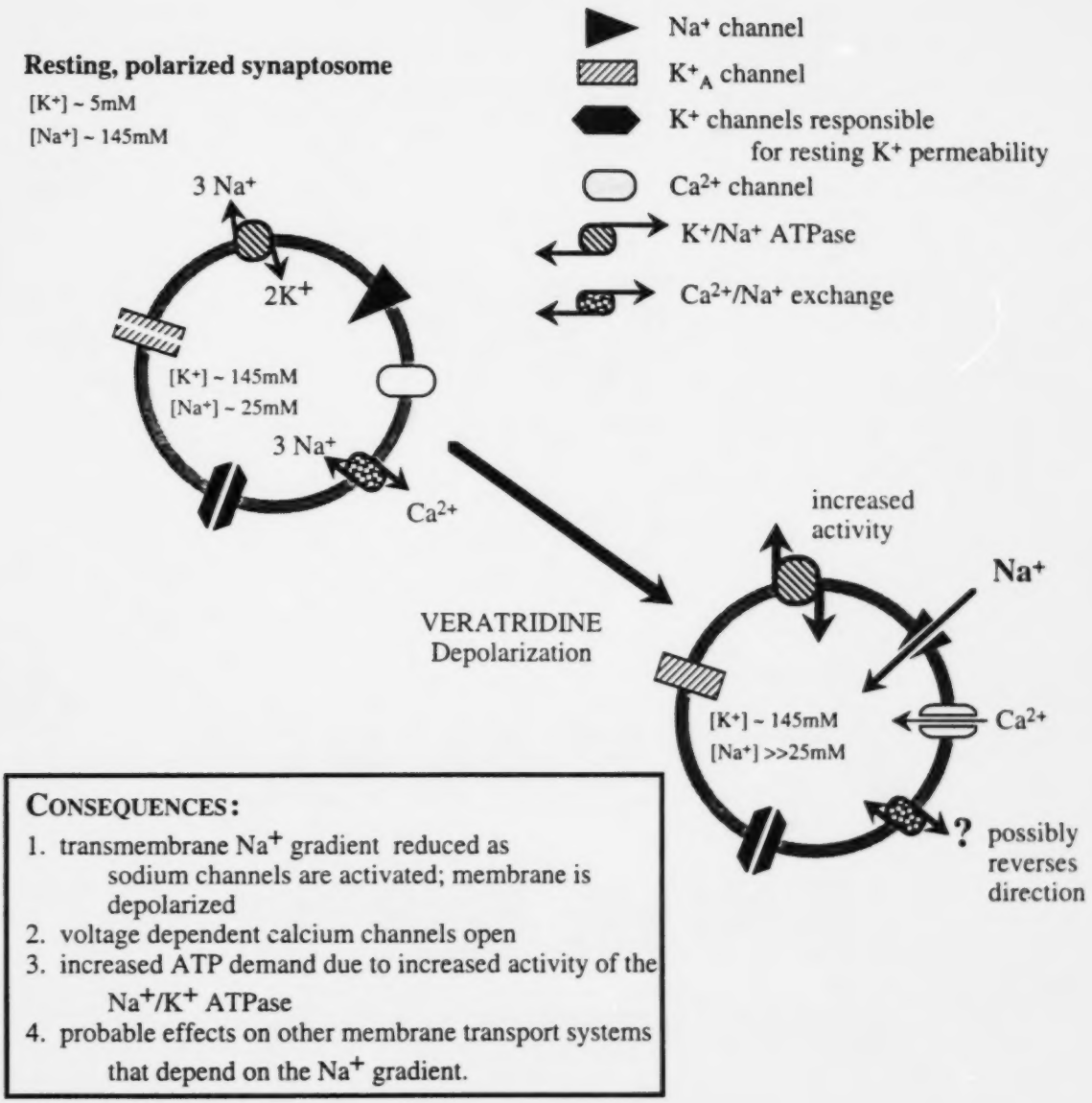


Figure 1.2 A schematic diagram of the consequences in synaptosomes of membrane depolarization induced by veratridine. Activation of sodium channels by veratridine leads to an influx of Na^+ and depolarization according to the parameters of the Nernst equation. The Na^+ gradient across the membrane is reduced and this is likely to have the additional consequences of altered or possibly reversed action of Na^+-linked ion exchange and membrane transport processes.

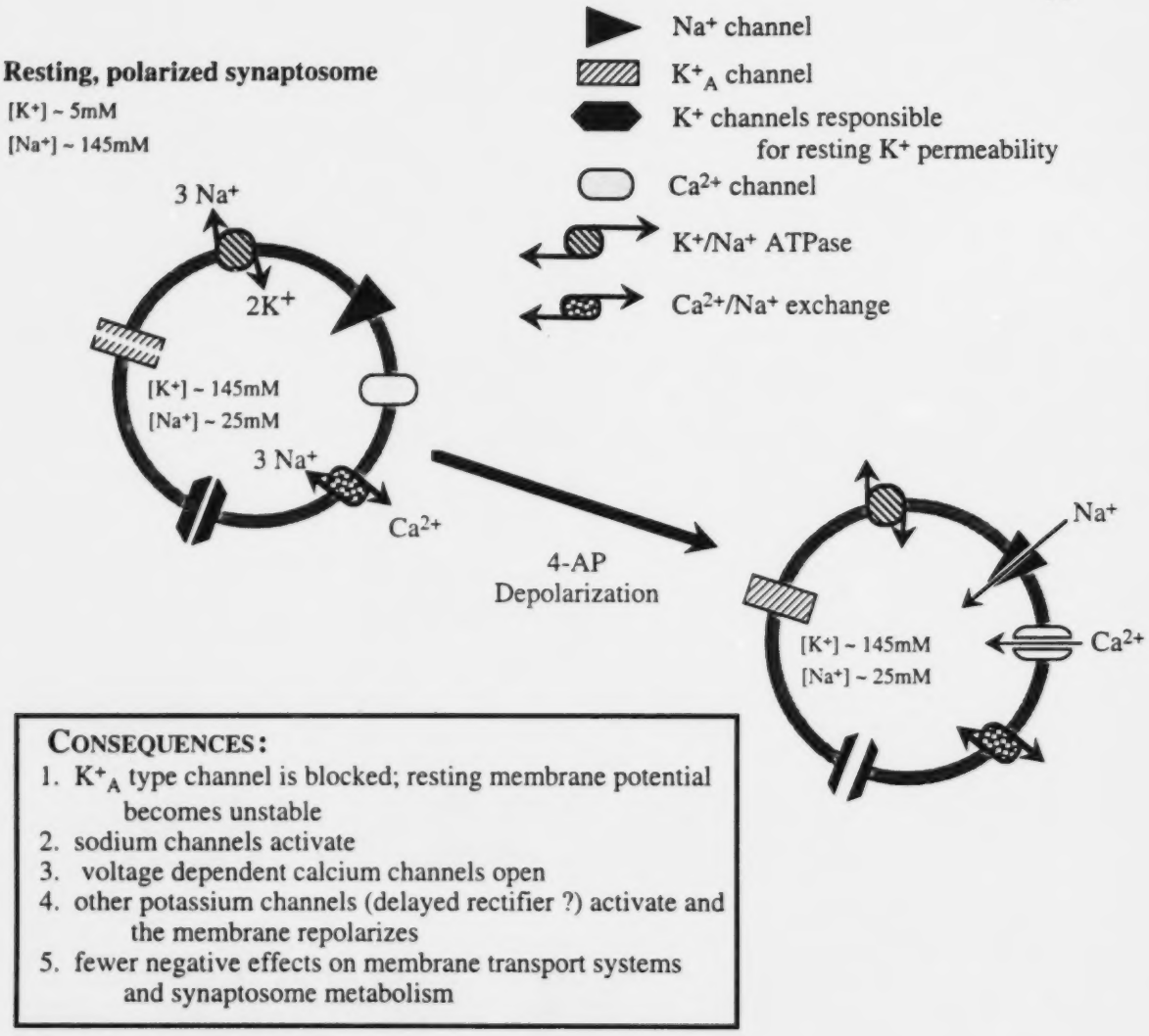


Figure 1.3 A schematic diagram of the consequences in synaptosomes of 4-AP induced depolarization. 4-AP blocks a subset of low-voltage activated presynaptic potassium channels that help to maintain the resting membrane potential. Block of these channels leads to instability in the resting membrane potential such that it may drift upward to the threshold potential for activation of sodium channels. The depolarization induced by the influx of Na^+ is limited by sodium channel inactivation and subsequent activation of additional potassium channels (analogous to delayed rectifier channels). This cycle may repeat, leading to repetitive action potentials. It is thought that neither K^+ nor Na^+ membrane gradients are seriously compromised by depolarization with 4-AP relative to K^+ or veratridine.

The Use of Fura-2 to Monitor $[Ca^{2+}]_i$: Synaptosome vesicles are relatively small, on the order of 1 μm in diameter (Whittaker, 1984), and it is not yet possible to record from them electrophysiologically. In order to monitor the influx of Ca^{2+} into synaptosomes, $^{45}Ca^{2+}$ or fluorescent dyes have been widely employed (Nachshen, 1984; Komulainen & Bondy, 1987; Dunn, 1988; Lundy *et.al.*,1992; Lentzner *et.al.*, 1992; Hewett & Atchison, 1992; Romano-Silva *et.al.*, 1994; Bartschat & Rhodes, 1995).

Fluorescent methods of measuring intracellular Ca^{2+} levels utilize Ca^{2+}-chelating molecules such as Quin-2, Indo-1 and Fura-2 that are based on the structure of EGTA and undergo a measurable change in fluorescent properties on binding Ca^{2+} (Tsien, 1988). Fura-2 has been among the most popular of these dyes due to its greater fluorescence and sensitivity to Ca^{2+} over Mg^{2+} (Thomas & Delaville, 1991). Fura-2 also undergoes a shift to a shorter excitation peak on complexing with Ca^{2+} so that monitoring the fluorescence at two excitation wavelengths can be used to make an estimate the actual $[Ca^{2+}]$. The K_d of Fura-2 for Ca^{2+} is approximately 225 nM (Grynkiewicz *et.al.*, 1985). Fluorescent indicators of $[Ca^{2+}]_i$ are sensitive and relatively simple to use; however the fluorescence signal may be affected by a variety of factors. The most important limitations of the use of such dyes, particularily for estimating $[Ca^{2+}]_i$ in synaptosome studies, include compartmentation of the dye into non-terminal vesicles; variable/inadequate loading and de-esterfication of the membrane-permeant form of the dye; or interactions of the dye with intracellular proteins and other components. Appropriate control and calibration studies reduce the impact of these factors on the $[Ca^{2+}]$ measurements (Roe *et.al.*,1990; Thomas & Delaville, 1991; Yates *et.al.*, 1992; Neher, 1995). In addition, the Fura-2 fluorescence signal, like other measurements of synaptosomal function, represents the response of the population of terminals.

Synaptosomal Studies of Ca^{2+} Influx and Neurotransmitter Release: Synaptosomal preparations carry high affinity binding sites for radiolabelled dihydropyridines (Dunn, 1988), yet in many studies it has been reported that these drugs have little effect on depolarization-induced Ca^{2+} uptake (Lundy *et.al.*, 1991; Pocock & Nicholls, 1992;

Suszkiw *et.al.*, 1986; 1989). In most, but not all, studies, voltage-dependent calcium channels in mammalian synaptosomes have been shown to be insensitive to ω-conotoxin GVIA (Hillyard *et.al.*, 1992; Lentzner *et.al.*, 1992; Lundy *et.al.*, 1991; Mintz *et.al.*, 1992; Pocock & Nicholls, 1992; Suszkiw *et.al.*, 1989), although this toxin potently blocks Ca^{2+} uptake by avian and amphibian synaptosomes (Lundy *et.al.*, 1991; Suszkiw *et.al*, 1987; Venema *et.al.*, 1992). $^{45}Ca^{2+}$ uptake by mammalian synaptosomes does, however, appear to be sensitive to natural or synthetic FTX (Uchitel *et.al.*, 1992) and also to be blocked by venom from the related funnel web spider, *Hololena curta* (Lundy *et.al.*, 1992). Recently, a *Conus magus* toxin, ω-conotoxin MVIIC, has been shown to block $^{45}Ca^{2+}$ uptake by rat brain synaptosomes. This toxin also inhibited P-type currents in cerebellar Purkinje cells in addition to an ω-conotoxin GVIA-insensitive current in CA1 hippocampal pyramidal cells (Hillyard *et.al.*,1992).

It is quite likely that the distribution of calcium channels at the nerve terminal is different from the cell body, and thus it is desirable to obtain even indirect measurements of presynaptic modulatory effects. Ca^{2+} influx in synaptosomes is increased on activation of protein kinase C (Bartschat & Rhodes, 1995) and inhibited in the presence of agonists of $GABA_B$ receptors (Tareilus *et.al.*, 1994b). Several groups have reported changes in neurotransmitter release from synaptosomes in the presence of agonists for $GABA_B$, ATP and other presynaptic autoreceptors (Herrero *et.al.*, 1992b; Coffey *et.al.*, 1993). Modulation of voltage-sensitive calcium channels through second messenger systems adds considerable complexity, but also considerable potential for very "fine tuning" of the channel activity.

Several reports of 4-AP stimulated Ca^{2+} influx and transmitter release from cortical synaptosomes have been published (Agoston *et.al.*, 1983; Tapia *et.al.*, 1985; Heemskerk *et.al.*, 1991; Herrero *et.al.*, 1991; Chapell & Rosenberg, 1992; Nichols *et.al.*, 1994; Terrian, 1995). Differential kinetics of Ca^{2+} influx when the depolarizing stimulus is 4-AP or high K^+ have been observed with an overall greater uptake of Ca^{2+} in the presence of high K^+ (Herrero *et.al.*, 1991; McMahon & Nicholls, 1991; Tibbs *et.al.*, 1989b). Calcium-dependent endogenous glutamate release is slightly slower

following 4-AP depolarization (Herrero *et.al.*, 1991) and while synaptosomal release of tritiated neurotransmitters occurs with 4-AP stimulation, this is greater in the presence of elevated external K^+ (Chapell & Rosenberg, 1992). In addition, several authors have reported that the phosphorylation states of presynaptic proteins are differentially affected by 4-AP or high [K^+] depolarization (Heemskerk *et.al.*, 1991; Nichols *et.al.*, 1994). The distinct effects of these stimulating protocols support the hypothesis that 4-AP and elevated [K^+] depolarize the membrane in different ways. If block of K^+ channels by 4-AP results in depolarization through a mechanism by which the membrane potential repeatedly spikes, one could expect that the functional measurements of Ca^{2+} fluxes through voltage sensitive calcium channels may differ from those observed under a one-time clamped depolarization protocol. In particular, L-type calcium channels inactivate slowly and effects of antagonists at these channels have been shown to be both frequency and use-dependent (Triggle, 1991; Uski *et.al.*, 1994). Thus depolarization by 4-AP may result in a Ca^{2+} influx that is more sensitive to block by verapamil and dihydropyridines.

Purpose of Current Work:

The underlying hypothesis of the current work was that pharmacologically distinct calcium channels are present at the presynaptic terminal and may be identified as such. The specific objectives were as follows:

1. To develop a stopped-flow fluorimetric technique to measure rapid depolarization-induced Ca^{2+} influx in Fura-2 loaded synaptosomes.

2. To characterize, by pharmacological methods, the voltage-dependent calcium channels responsible for the Ca^{2+} influx in depolarized synaptosomes.

3. To study the effects of different methods of depolarization on the pharmacology of the presynaptic Ca^{2+} signal.

4. To investigate the modulation of Ca^{2+} influx by presynaptic mechanisms.

5. To investigate the possibility that antibodies directed against presynaptic calcium channels play a role in the autoimmune disease amyotrophic lateral sclerosis.

6. To evaluate different methods of measuring neurotransmitter release on rapid time scales in order to determine the functional significance of subtype-specific Ca^{2+} influx.

Although synaptosomes have been very useful in contributing to our understanding of many presynaptic mechanisms, in most of the studies cited above, the interpretation of Ca^{2+} uptake and neurotransmitter release data has been complicated by the lack of time resolution in the assays employed. Measurements of $^{45}Ca^{2+}$ influx in response to depolarization of the synaptosome membrane rarely have the temporal resolution to approach the physiological time scales of presynaptic events. The importance of measuring flux responses on much more rapid (millisecond) timescales has been recognized. Some recent reports (Lentzner *et.al.*, 1992; Tareilus & Breer, 1992; Tareilus *et.al.*, 1993; 1994a; 1994b; Thomas *et.al.*, 1994; Bartschat & Rhodes, 1995; this thesis) describe the use of entrapped Ca^{2+}-sensitive fluorescent dyes to measure subsecond changes in $[Ca^{2+}]_i$. Although this time resolution is not fast enough to resolve the Ca^{2+} transients that are involved in triggering the release process, real time measurements of changes in cytosolic Ca^{2+} levels may be made.

A stopped-flow fluorescence technique to measure rapid depolarization-induced changes of $[Ca^{2+}]_i$ has been developed and has been used to investigate presynaptic calcium channel function in rat cortical synaptosomes (Thomas *et.al.*,1994). The calcium channel pharmacology of the Ca^{2+} influx, resulting from depolarization by elevated $[K^+]_o$ or by 4-AP, has been investigated using this system. The voltage-dependence and inorganic cation sensitivity of the flux was evaluated, as well as the sensitivity of the presynaptic Ca^{2+} flux to other voltage sensitive calcium channel antagonists including 1,4-dihydropyridines, ω-conotoxin GVIA, components from the venom of the funnel web spider, *Agelenopsis aperta*, and ω-conotoxin MVIIC. A number of other pharmacological agents including aminoglycoside antibiotics and various agonists and

antagonists of proposed presynaptic receptors were also examined for an effect on presynaptic Ca^{2+} influx.

In order to investigate the possibility that an autoimmune response to presynaptic calcium channels is involved in the pathogenesis of ALS, a study of the specific effects of purified IgG from ALS patients on synaptosomal calcium channels was carried out using this stopped-flow technique.

A number of approaches to measure neurotransmitter release on rapid timescales were investigated in order to correlate the pharmacology of the Ca^{2+} influx observed with the release of a specific transmitter. The advantages and disadvantages of different experimental approaches are discussed. The most reliable technique involved loading the synaptosomes with radiolabelled GABA (^{3}H) and glutamate (^{14}C) and monitoring the release of the transmitters following vacuum filtration. Pre-loading synaptosomes with two transmitters is a relatively novel aspect of this approach.

The studies described in this thesis were undertaken in order to further clarify the role of voltage-dependent Ca^{2+} influx in presynaptic function. The combination of rapid measurements of Ca^{2+} signals using stopped-flow fluorimetry and an extensive series of potential modulators of presynaptic Ca^{2+} influx will meaningfully extend current understanding of presynaptic calcium channel pharmacology.

Chapter Two
General Methods

Buffers and Drug Solutions: All buffers were prepared with distilled, Millipore-filtered water (dH_2O) and reagent grade chemicals and were pH-adjusted with NaOH unless otherwise indicated.

BUFFERED SOLUTIONS

0.32M Sucrose:	O.32 M sucrose, 5 mM Hepes-Na^+ (pH 7.4)
0.8M Sucrose:	O.8 M sucrose, 5 mM Hepes-Na^+ (pH 7.4)
Ca^{2+}-free Choline:	10 mM Hepes-Na^+, 145 mM choline chloride, 5 mM KCl, 1.2 mM $MgCl_2$, 5.5 mM glucose (pH 7.4)
Ca^{2+}-free Sodium:	10 mM Hepes-Na^+, 145 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 5.5 mM glucose (pH 7.4)
Nondepolarizing Choline:	10 mM Hepes-Na^+, 145 mM choline chloride, 5 mM KCl, 1.2 mM $MgCl_2$, 0.1mM $CaCl_2$, 5.5 mM glucose (pH 7.4)
Nondepolarizing Sodium:	10 mM Hepes-Na^+, 145 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 0.1mM $CaCl_2$, 5.5 mM glucose (pH 7.4)
K^+-depolarizing Choline:	10 mM Hepes-Na^+,1.2 mM $MgCl_2$, 0.1mM $CaCl_2$, 5.5 mM glucose (pH 7.4) with variable KCl and choline chloride added to give a final total concentration of 150mM, as indicated for a given experiment.
K^+-depolarizing Sodium:	10 mM Hepes-Na^+,1.2 mM $MgCl_2$, 0.1mM $CaCl_2$, 5.5 mM glucose (pH 7.4) with variable KCl and NaCl added to give a final total concentration of 150mM, as indicated for a given experiment.
1mM 4-Aminopyridine:	10 mM Hepes-Na^+, 1mM 4-AP, 145 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 0.1mM $CaCl_2$, 5.5 mM glucose (pH 7.4).
Phosphate saline:	137mM NaCl, 3mM KCl, 12mM sodium phosphate (pH 8.0).
Lithium Acetate:	0.1M lithium acetate, pH adjusted to 5.5 with HCl

Borate buffer: 0.40 M di-sodium tetraborate; pH adjusted to 10.4 with 6N NaOH

STOCK SOLUTIONS AND SOURCES

Fura 2-AM: 1 mM in anhydrous dimethylsulphoxide
Molecular Probes Inc., Eugene, OR

diSC$_3$(5): 3,3'-dipropylthiodicarbocyanine
0.5 mM in ethanol
Molecular Probes Inc., Eugene, OR

Biorad assay: BioRad Laboratories, Richmond,CA

Cations: 10 mM in nondepolarizing buffer
Sigma Chemical Co., St. Louis, MO

± BayK 8644: 10 mM in ethanol
- gift of Dr. A. Scriabine,
Miles Inc., West Haven CT.

± nitrendipine: 10 mM in ethanol
- gift of Dr. A. Scriabine,
Miles Inc., West Haven CT.

± nimodipine: 10 mM in ethanol
- gift of Dr. A. Scriabine,
Miles Inc., West Haven CT.

± verapamil-HCl: 10 mM in dH_2O
Sigma Chemical Co., St. Louis, MO

ω-conotoxin GVIA: 1 mM in dH_2O
Bachem Inc, Torrance, CA or
Cambridge Research Biochemicals, Cheshire UK
or Peninsula Laboratories, CA

***Agelenopsis aperta* venom:** 8.25mg/mL dH_2O
Spider Pharm, Feasterville, PA

ω-Agatoxin IVA:	1 mM in dH_2O - gifts of Dr. M.E. Adams and Pfizer Inc., Groton CT. -Research Biochemicals Inc., Natick MA.
FTX:	1:10 dilution in dH_2O - gift of Drs. R.R. Llinás and B. Cherksey
sFTX:	100mM in dH_2O - gift of Drs. R.R. Llinás and B. Cherksey
ω-conotoxin MVIIC:	1 mM in dH_2O Bachem, Torrance, CA
2-chloroadenosine:	10 mM in dH_2O Sigma Chemical Co., St. Louis, MO
bekanamycin:	10 mM in nondepolarizing buffer Sigma Chemical Co., St. Louis, MO
neomycin:	10 mM in nondepolarizing buffer Sigma Chemical Co., St. Louis, MO
gentamycin:	10 mM in nondepolarizing buffer Sigma Chemical Co., St. Louis, MO
streptomycin:	10 mM in nondepolarizing buffer Sigma Chemical Co., St. Louis, MO
baclofen:	10 mM in dH_2O -gift from CIBA-GEIGY Ltd., Basel Switzerland
neuropeptide Y:	100 μM in dH_2O INRS, Pt. Claire PQ
neurosteroids:	10 mM in ethanol Sigma Chemical Co., St. Louis, MO
Protein A Sepharose:	Sigma Chemical Co., St. Louis, MO
luminol:	5-amino-2,3-dihydro-1,4-phthalazinedione 2mg/mL dH_2O Sigma Chemical Co., St. Louis, MO

choline oxidase:	EC 1.1.3.17 250 units/mL dH_2O Sigma Chemical Co., St. Louis, MO
horseradish peroxidase:	EC 1.11.1.7 4mg/mL dH_2O Sigma Chemical Co., St. Louis, MO
acetylcholine esterase:	EC 3.1.1.7 1000 units/ml dH_2O Sigma Chemical Co., St. Louis, MO
glutamate dehydrogenase:	EC 1.4.1.3 2.4 mg/ml nondepolarizing buffer Sigma Chemical Co., St. Louis, MO
$NADP^+$:	20 mM in dH_2O Sigma Chemical Co., St. Louis, MO
Gabase:	5 unit/mL in 0.075 M phosphate, 25% glycerol; pH 7.2 Sigma Chemical Co., St. Louis, MO
OPA	o-phthaldialdehyde, Aldrich Chemical Company Inc., Milwaukee WI
BRIJ-35	30% w/v, Sigma Chemical Co., St. Louis, MO
methanol	HPLC grade, Optima Fisher Scientific Ltd.
β-mercaptoethanol	BDH Chemicals Ltd., Poole, UK.
amino-oxyacetic acid:	10 mM in Ca^{2+}-free sodium buffer Sigma Chemical Co., St. Louis, MO
[^{3}H]GABA:	1 mCi/mL in 0.01N HCl New England Nuclear, Dupont
[^{14}C] glutamate:	0.1 mCi/mL in 2% ethanol New England Nuclear, Dupont

Preparation of Synaptosomes from Rat Cerebral Cortex

Synaptosomes were prepared from the cerebral cortices of male Sprague-Dawley rats (100-150 g) by a modification of the protocol of Ray *et. al.* (1978). All procedures were carried out at 0 - 4°C. The rats were decapitated and the whole brain was carefully removed. Following dissection to remove the cerebellum, hippocampal and mid-brain tissues, the cortices were coarsely chopped and homogenized in 0.32 M sucrose buffer (5 mL/brain) using a Potter S glass-teflon homogenizer at 600 rpm. The homogenate was centrifuged at 1100 x g_{max} in a Sorvall SS34 rotor for 10 min, after which the supernatant was kept on ice while the pellets were resuspended in sucrose buffer (5 mL/brain) and centrifuged as before. The supernatants were combined and centrifuged at 12000 x g_{max} for 20 min. The supernatant was discarded and the pellets, less the central darker portion, were resuspended in 0.32 M sucrose buffer (10 mL/brain). A two layer sucrose gradient was prepared by layering 10 mL of the membrane preparations on top of 20 mL 0.8 M sucrose buffer in Beckman SW28 tubes. The gradients were centrifuged for 30 min at 9100 x g_{max} in a Beckman SW28 rotor. The 0.32 M sucrose layer and interface were removed by aspiration and the 0.8 M layer was collected and diluted dropwise with 2.5 volumes of Ca^{2+}-free choline buffer. The synaptosomes were collected by centrifugation for 6 min at 20200 x g_{max} in a Sorvall SS34 rotor and were resuspended in Ca^{2+}-free choline buffer to give a concentration of 2-4 mg protein/mL as measured by the BioRad assay. Aliquots of 500 μL or 1 mL were frozen on dry ice and stored at -86°C until use. Such freezing has been previously shown to have little effect on synaptosome viability or the results of $^{45}Ca^{2+}$ uptake studies (Dagani & Erecińska, 1987; Dunn, 1988).

Monitoring viability by membrane potential measurements: Synaptosome viability was assessed by their ability to maintain a membrane potential as monitored using the fluorescent potential sensitive dye, $diSC_3(5)$. A 5 μL aliquot of 500 μM dye in ethanol was added to 2.45 mL of nondepolarizing buffer or high K^+ depolarizing buffer, or 1 mM 4-AP buffer in disposable polystyrene fluorescence cuvettes. The final concentration of the dye was 1 μM and the ethanol concentration was 0.2%. The fluorescence was

measured using a Perkin-Elmer MPF4 fluorimeter with excitation and emission wavelengths of 622 nm and 670 nm respectively. The samples were equilibrated at 37°C and synaptosomes were added, from a thawed aliquot, to a final protein concentration of 50 μg/mL. The fluorescence change of each sample was measured at approximately 10 minute intervals for up to 2 hours. The fluorescence changes of $diSC_3(5)$ observed on mixing synaptosomes with depolarizing or nondepolarizing buffers were consistent with the presence of an electrical potential difference across the membrane (Blaustein & Goldring, 1975). It was routinely found that a good synaptosome preparation would maintain its membrane potential for over 1 hour at 37°C and often for more than 2 hours.

Fluorescence Measurements

Loading of Synaptosomes with Fura-2: The optimal conditions for loading of the synaptosomes with Fura 2 prepared following the above procedure, were established in our laboratory. In preliminary experiments, the concentration of free Ca^{2+} within the synaptosomes was measured using the procedures of Grynkiewicz and co-workers (Grynkiewicz *et.al.*, 1985) It was found that when synaptosomes were prepared in buffer containing 1.2 mM $CaCl_2$ as we have used in previous studies (Dunn, 1988) and loaded with Fura-2 as previously described (Komulainen & Bondy, 1987), the resting synaptosomal free Ca^{2+} concentration lay in the range of 400 - 500 nM. Since this is considerably higher than the K_d of the Fura-2-Ca^{2+} complex (224 nM at 37°C; Grynkiewicz *et.al.*, 1985), this was unsuitable for measuring transient increases in cytosolic Ca^{2+} levels. Subsequent evaluation of $[Ca^{2+}]_i$ in synaptosomes prepared in nominally Ca^{2+}-free medium indicated a resting Ca^{2+} concentration of 100-200 nM. (Thomas *et.al.*,1994)

Since synaptosomes remain viable for only limited times (Verhage *et.al.*,1988), it was necessary to load the dye both rapidly and efficiently so that the synaptosomes retained their viability over the time course of subsequent experiments and that fluorescence changes were readily and reliably measurable. In previous studies, it has

been shown that the ratio of Fura-2 fluorescence using excitation at 340 nm to excitation at 380 nm can be taken as a measure of dye-loading efficiency (Komulainen & Bondy, 1987). Using this method it was found that the efficiency of loading at 37°C is much greater than at 25°C, but even at the higher temperature, loading is incomplete after 1 hour. After 30 minutes however, there was sufficient Fura-2 loading that intrasynaptosomal free Ca^{2+} concentrations could be reliably measured. Prior to all stopped-flow experiments, a 1 mM stock solution of Fura-2AM was prepared in anhydrous dimethylsulphoxide. Synaptosomes were thawed and diluted with an equal volume of Ca^{2+}-free choline or sodium buffer as appropriate for the experiment. Fura-2AM was added to a final concentration of 5 μM (< .5% DMSO) and the synaptosomes were incubated, with shaking, for 30 minutes at 37°C. To remove residual external Fura-2AM, the loaded synaptosomes (400 μl) were applied to 2 mL Sephadex G-50-300 columns that had been equilibrated in Ca^{2+}-free buffer and pretreated with synaptosomal membranes to saturate non-specific binding sites. The columns were immediately centrifuged in a bench top centrifuge for 20 sec. The synaptosomes collected in the eluate were diluted five-fold and $CaCl_2$ was added back to give a final Ca^{2+} concentration of 0.1 mM. The synaptosomes were equilibrated for at least 10 min at room temperature prior to stopped-flow experiments.

Stopped-flow Fluorescence Measurements of Changes in Intrasynaptosomal Ca^{2+}: Kinetic measurements were made using an Applied Photophysics Ltd. (Leatherhead, U.K.) SF.17MV Microvolume Stopped-Flow Fluorimeter (Figure 2.1). Prior to mixing, the synaptosomes and non-depolarizing or depolarizing buffer (containing isoosmotic substitution of choline or sodium by K^+) were equilibrated to 37°C for 3-5 min in the stopped-flow instrument. Where appropriate, drugs or toxins were prepared as stock solutions in ethanol or distilled water and were added to both the synaptosome suspension and appropriate mixing buffer at least 15 minutes prior to making stopped-flow recordings. The final concentration of ethanol did not exceed 0.02%. In any one experiment, control measurements i.e. mixing with non-depolarizing or depolarizing buffers were carried out at random throughout the experiment to verify that observed

drug effects were not due to a decrease in synaptosomal responses as a function of time. Excitation at 390 nm was obtained with a grating monochromator placed between the 150W xenon arc source and the observation cuvette. In some experiments a Corning 0-52 filter was placed at the monochromator exit slit to reduce stray light below 350 nm. Fluorescence was recorded using a high-pass (>420 nm) GG420 or GG495 emission filter (Melles Griott, Irvine, CA) and data were collected and analyzed using an Archimedes computer and the Applied Photophysics Ltd. kinetic software package.

Reaction traces were recorded over a period of 1 sec and, in most experiments, a dual time base was used such that half of the 400 data points recorded were from the first 100 msec. This provided increased resolution of rapid responses. The traces were analyzed using a single exponential model:

$$\text{Fluor} = \text{Amp}_1 \times \exp(-k_1 t) + \text{Amp}_{eq}$$

where Fluor is the recorded fluorescence level (in millivolts), Amp_1 and k_1 are the amplitude and rate constant of the observed fluorescence quench respectively, and Amp_{eq} is the equilibrium fluorescence level at the end of the reaction. A single exponential model is a reasonable approximation of the fluorescence change, however the Ca^{2+} signal arises from several contributing processes.

Drug or toxin concentration-dependent effects on the fluorescent signal are presented as plots of the ligand dose versus the normalized, average Amp_1 response. Curve fitting was carried out using the competition curve algorithm provided in the software program InPlot4 (GraphPad Software Inc., ver. 4.03, 1992).

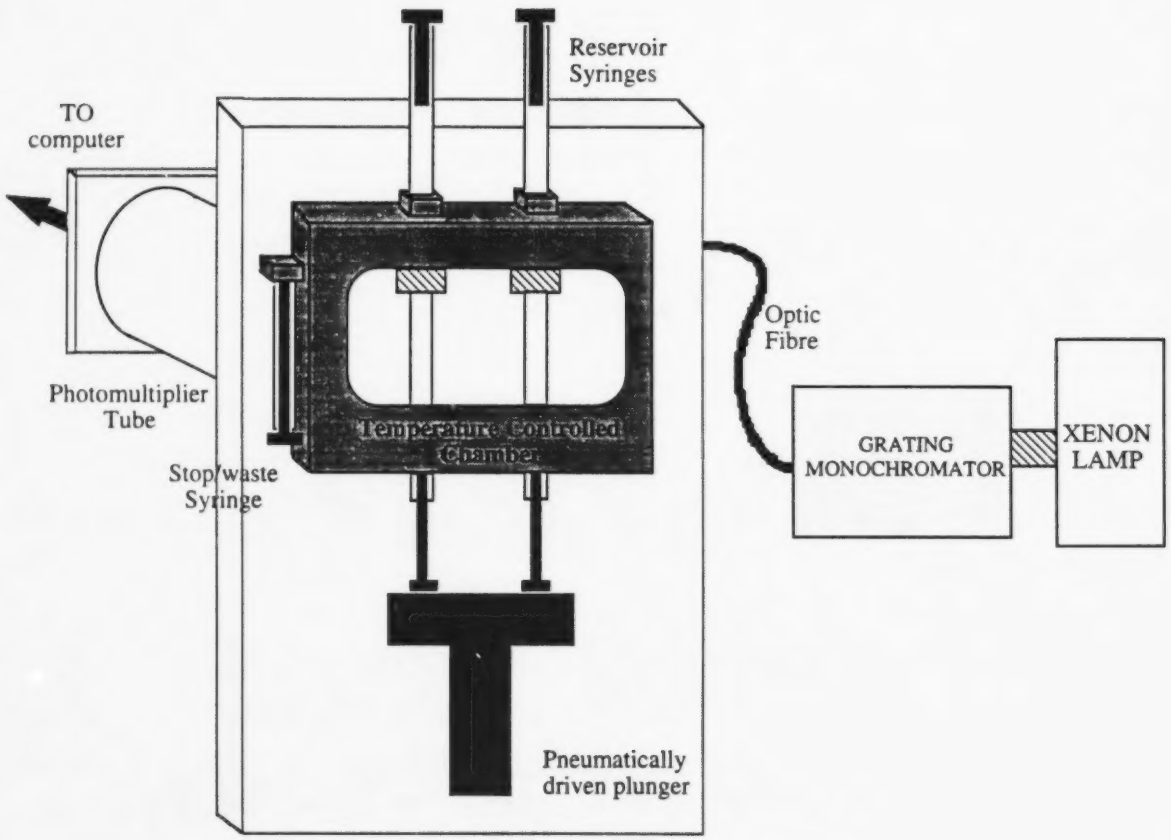


Figure 2.1 Schematic Diagram of the Stopped-flow Fluorimeter.

$[Ca^{2+}]_i$ Measurements: In order to quantitate the changes that occur in intrasynaptosomal $[Ca^{2+}]$ on depolarization, equilibrium fluorescence measurements were made according to the method of Koumulainen and Bondy (1987). Synaptosomes were loaded with Fura-2 as described above and the external $[Ca^{2+}]$ was adjusted to 0.1 mM. 1.5 mL aliquots of the dye-loaded synaptosomes were placed in quartz cuvettes. Excitation spectra were recorded from 300-400 nm using an emission wavelength of 510 nm. Following the recording of an initial spectrum, 1.5mL of depolarizing or nondepolarizing buffer were added and the excitation spectrum were recorded again. A range of K^+ concentrations, as well as 1 mM 4-AP, were tested for depolarization-induced changes in $[Ca^{2+}]_i$. In order to calibrate the signal as described by Grynkiewicz *et.al.* (1985), excitation spectra were recorded under each buffer condition following the addition of (1) 40 μM $MnCl_2$, (2) 2.5mM EGTA, (3) 22mM Tris/5% SDS and (4) 3mM $CaCl_2$. The $[Ca^{2+}]$i was calculated according to the following equation,

$$[Ca^{2+}]_i = \frac{(R_{Mn} - R_{min})}{(R_{max} - R_{mn})} \times \frac{SF_2}{SB_2} \times 224nM$$

where R is the ratio of fluorescence at excitation of 340 nm to that at 380 nm, specifically:

R_{mn} is the ratio following quench of external dye with $MnCl_2$,

R_{min} is the ratio following lysis by SDS, in the presence of EGTA

R_{max} is the ratio following addition of excess Ca^{2+},

SF_2 is the measured fluorescence at 380nm after addition of SDS and EGTA

SB_2 is the measured fluorescence at 380nm following addition of excess Ca^{2+}

and 224nM is the K_d of the Fura-2-Ca^{2+} complex (Grynkiewicz *et.al.*, 1985).

Isolation of IgG

Sample Collection: Blood samples were collected in accordance with the University of Alberta guidelines from eight ALS-diagnosed individuals and five non-neurological

control subjects. The sera was collected in vacuum tubes without additives, and immediately centrifuged to separate the blood cells and plasma. The plasma was removed and stored at -20°, or in the presence of 0.02% NaN_3 at 4°C until further purified.

Purification of IgG: The plasma was diluted 1:1 with phosphate buffered saline (PBS), pH 8.0 and applied to a 5 mL Protein A-Sephadex column. After the column was extensively washed with PBS, IgG was eluted with 0.1 M citric acid (pH 3.0) at a flow rate of 0.5 mL/min. One or two minute fractions were collected and the pH was immediately neutralized with 2M Tris. Absorbance readings at 280nm were monitored and the fractions containing significant amounts of protein were prepared for SDS polyacrylamide gel electrophoresis analysis (Laemmli, 1970). Fractions with a high protein content and high purity of IgG, as indicated by the SDS PAGE results, were combined. The combined fractions were dialysed for 48 hours at 4°C against the calcium-free buffer used in stopped-flow experiments. Protein content was measured and the samples were stored at 4°C in 0.02% NaN_3 until use.

Use of Purified IgG in Stopped-flow Fluorimetry Experiments: Synaptosomes were loaded with Fura-2 as described above. Purified IgG was added to both the synaptosome suspension and appropriate mixing buffer to give final IgG concentrations between 1μg/mL and 1mg/mL. The samples were equilibrated at room temperature for at least 20 minutes prior to measurement of Ca^{2+} influx.

In each experiment IgG was tested for effects on Ca^{2+} influx under both depolarizing and control conditions. Control measurements, in which IgG-free buffers were used, were collected at random throughout the experiments. The limited amount of patient IgG available meant that, for most cases, only two or three experiments per ALS sample and depolarization condition could be completed. Thus the repeated control measurements were used to correct for possible time-dependent changes occurring during the course of an experiment.

Heat-inactivated IgG, prepared by heating patient or control samples of IgG to

95°C for 20 minutes, was tested in both stopped-flow experiments and in control membrane potential measurements to monitor possible effects of the protein on synaptosome viability. Additional control studies confirmed that the presence of 0.002% NaN_3, the maximum concentration used in this study, had no effect on the stopped-flow signal.

Stopped-flow traces were collected and analysed as described above. The best fit value for the Amp_1 measurement was used in subsequent data analysis. In all experiments, the amplitude measurements taken under the various conditions were normalized to those of control traces collected in the absence of added IgG.

Neurotransmitter Release Studies

Several different methods of measuring neurotransmitter release from synaptosomes were investigated. The methodology of these transmitter release assays are presented in Chapter 6 of this thesis.

Chapter Three

Pharmacological Characterization of Presynaptic Ca^{2+} Signals Following 27.5 mM KCl-Induced Depolarization

RESULTS

Depolarization-Dependent Changes in Intrasynaptosomal Ca^{2+}: The Ca^{2+}-sensitive fluorescent dye, Fura-2, was used to monitor changes in intrasynaptosomal $[Ca^{2+}]$. Upon the binding of Ca^{2+}, the fluorescence excitation maximum of Fura-2 shifts from about 390 nm to 340 nm (Grynkiewicz *et.al.*, 1985). An increase in $[Ca^{2+}]_i$ is thus accompanied by an increase in the fluorescence of entrapped Fura-2 when excited at 340 nm and a quench when excited at 390 nm. This shift in the excitation spectrum of Fura-2 can be used to quantitate $[Ca^{2+}]_i$ (Grynkiewicz *et.al.*,1985; Tsien, 1988). Under equilibrium conditions, estimates of the $[Ca^{2+}]_i$ of synaptosomes prepared as described in Chapter 2, were made using a Perkin-Elmer MPF4 fluorimeter. In nondepolarizing buffer ($[K^+]$=5 mM; $[Ca^{2+}]$=0.1 mM) the $[Ca^{2+}]_i$ was calculated to be 195 $\pm$ 17 nM (n=14 separate determinations). Presentation of 27.5 mM KCl, by iso-osmotic replacement of choline chloride, resulted in a depolarization-induced increase in $[Ca^{2+}]_i$ to 235 $\pm$ 26 nM (n=11 separate determinations).

Measurements of real time changes in the intrasynaptosomal Fura-2 fluorescence signal have been monitored by stopped-flow fluorimetry. Figure 3.1 illustrates a typical stopped-flow recording when Fura-2 loaded synaptosomes were rapidly mixed with an equal volume of a non-depolarizing (5 mM K^+) buffer or with a depolarizing buffer (containing 50 mM K^+ to give a final $[K^+]_o$ of 27.5 mM). Depolarization led to a rapid increase in synaptosomal free Ca^{2+} levels which was accompanied by a quench in the Fura-2 fluorescence excited at 390 nm. Depolarization also resulted in an increase in fluorescence when the excitation wavelength was 340nm but a better signal-to-noise ratio was routinely observed at 390 nm due to lesser light scattering by the synaptosomes. This latter wavelength was therefore used in all the stopped-flow experiments described below.

Attempts were made to measure free intrasynaptosomal Ca^{2+} concentrations under the stopped-flow conditions but, in agreement with previous reports (Lentzner *et.al.*, 1992; Yates *et.al.*, 1992) the results showed a high degree of variability. This is due mainly to the difficulty in accurately determining values for the maximum and minimum fluorescence of Fura-2 in the presence of synaptosomes permeablized with detergent (25 μM digitonin). Although these values were variable, the voltage-dependence of the observed fluorescence signal amplitude was very consistent from experiment to experiment. It is therefore assumed that the magnitude of the signal reflects similar changes in free Ca^{2+} concentration to those measured under equilibrium conditions (see above).

In further support of the validity of monitoring $[Ca^{2+}]$ changes at a single wavelength, Neher (1995) has considered the relationship of free Ca^{2+}, dye-bound Ca^{2+} and endogenously buffered Ca^{2+} to the observed fluorescent signal. It was found that the concentration of the indicator dye needs to be high enough to "outcompete" the buffering by Ca^{2+}-binding cellular components in order for the fluorescence change observed to be proportional to changes in free Ca^{2+} levels. Indeed some recent work has suggested that attempts to precisely quantitate $[Ca^{2+}]_i$ changes with ratiometric fluorescent dyes require careful consideration of the contribution of these different buffering factors and the recognition of the perturbations introduced to the system by use of a Ca^{2+}-chelating agent (Regher & Atluri, 1995; Neher, 1995).

Voltage-Dependence of Changes in Intrasynaptosomal Ca^{2+}: The effects of external K^+ concentration on the kinetics of changes in synaptosomal Fura-2 fluorescence are shown in Figure 3.2. The magnitude of the fluorescence quench increased with increasing external K^+ (with iso-osmotic replacement of choline chloride). The rates, however, showed little voltage-dependence and the reaction traces could be reasonably fit by a single exponential model with an apparent rate constant of 10-15 sec^{-1}.

In experiments similar to those previously described (Dunn, 1988), the fluorescent voltage-sensitive dye, $diSC_3(5)$, has been used to show that the magnitude of the membrane potentials generated by transmembrane K^+ gradients display the expected

dependence on external K^+. Using an estimated membrane potential of -80 mV for cortical synaptosomes under resting conditions (McMahon & Nicholls, 1991), membrane potentials have been calculated from the Nernst equilibrium potential for K^+ (i.e. $E_m \approx E_K = 61.54 \times \log\{[K^+]_o/[K^+]_i\}$). These values are indicated in Figure 3.2 for each external K^+ concentration investigated. The results show that the threshold for activation of the observed response is below -70 mV and the response is maximal above -40 mV. These potentials should, however, be considered only as approximations since it is not possible to directly measure the true membrane potential in isolated synaptosomes (Dunn, 1988; McMahon & Nicholls, 1991). In the experiments on calcium channel blockers described below, an intermediate KCl concentration (27.5 mM) predicted to generate a membrane potential of -32 mV, has been used.

Effects of Inorganic Calcium Channel Blockers: Several inorganic cations block calcium channels and different types of calcium channels have been reported to show a differential sensitivity to block by La^{3+}, Co^{2+} and Cd^{2+} (Miller & Fox, 1990; Suszkiw *et.al.*, 1989). The effects of these cations on Ca^{2+} influx in Fura-2 loaded synaptosomes is illustrated in Figure 3.3. All of the ions completely inhibited the depolarization-induced changes in intrasynaptosomal Ca^{2+} concentration. Co^{2+} appeared to have a fairly simple inhibitory effect with an IC_{50} of 4.7 μM. However, inhibition by La^{3+} was biphasic with about 70% of the response being suppressed with an IC_{50} of 133 nM and the remainder being blocked at $>$ 100 μM. The effects of Cd^{2+} were unusual in that there was an apparent enhancement of the depolarization-dependent response at sub-micromolar concentrations (5 independent experiments), although there was complete block at higher concentrations (IC_{50} approximately 4.8 μM).

Effect of Organic Voltage Dependent Calcium Channel Ligands: In order to investigate the possible role of different subtypes of calcium channels in mediating the observed changes in intrasynaptosomal Ca^{2+} concentration described above, the effects of dihydropyridines and verapamil, ω-conotoxin GVIA, ω-conotoxin MVIIC as well as crude venom and purified venom components from the funnel web spider *Agelenopsis*

aperta have been investigated. These drugs and toxins are relatively specific agents for the L-, N-, Q- or P-type voltage-dependent calcium channels that have been described (Miller & Fox, 1990; Milanjich & Ramachandran, 1995).

L-Type Blockers

Figure 3.4 illustrates the general ineffectiveness of L-type calcium channel ligands on the voltage-dependent changes of the Fura-2 signals. Nitrendipine had no discernible effect although nimodipine consistently blocked 10-15% of the influx at concentrations greater than 10 nM (8 separate experiments). The dihydropyridine agonist, Bay K8644, did not systematically alter the Ca^{2+} signal. Likewise, the phenyalkyamine, verapamil had no measurable effects on the influx.

N-Type Blockers

ω-Conotoxin GVIA is a peptide toxin from *Conus geopraphus* which has been shown to inhibit N-type calcium channels in a variety of species. The toxin was allowed to equilibrate with the Fura-2 loaded synaptosomes for at least 20 minutes at room temperature, in order to approach equilibrium at the lower concentrations, prior to depolarization. Although rat brain membranes carry binding sites with picomolar affinity for [^{125}I]ω-conotoxin GVIA (Barhanin *et.al.*, 1988), micromolar concentrations of this toxin blocked only about 15% of the observed response (Figure 3.5).

P/Q - Type Blockers

ω-Conotoxin MVIIC is another conotoxin, initally purified from a second species of fish-hunting snail, *Conus magus*. Figure 3.6 shows the effects of increasing concentrations of this toxin on the fluorescence signal resulting from synaptosome depolarization. High nanomolar concentrations of ω-conotoxin MVIIC were effective in blocking over 40% of the Ca^{2+} influx with an estimated IC_{50} value of 57.5 nM.

Venom from certain types of funnel web spiders has been shown to inhibit the function of voltage-dependent calcium channels. P-type channels are potently antagonized by the peptide, ω-agatoxin IVA, and the polyamine, FTX, both of which are

components from the venom of *Agelenopsis aperta*. Stopped-flow studies of presynaptic Ca^{2+} influx were carried out in the presence of crude venom, ω-agatoxin IVA, FTX and synthetic FTX (sFTX). The depolarization-induced changes in $[Ca^{2+}]_i$ were inhibited, in a dose-dependent manner, by crude venom from the funnel web spider, *Agelenopsis aperta* as shown in Figure 3.7. The venom was incubated with the Fura-2 loaded synaptosomes for at least 20 minutes prior to stopped-flow experiments in order to approach binding equilibrium at the lower venom concentrations. The dose dependence of block was complex, possibly biphasic and there was significant block ($\approx$60%) occurring at 300 ng/mL.

Subsequent studies with the peptide, ω-agatoxin IVA, or the purified polyamine fraction FTX demonstrated a potent inhibitory effect of these compounds on depolarization-induced Ca^{2+} influx. Figure 3.8 shows that ω-agatoxin IVA inhibited Ca^{2+} flux by 30% at submicromolar concentrations with an IC_{50} of 30 nM. As shown in Figure 3.9, the maximum inhibition by FTX was 40% (300 nL/mL) and the IC_{50} of this effect occurred with a dilution of 4 nL purified FTX per mL. In preliminary experiments, millimolar concentrations of synthetic FTX were found to partially block the fluorescence signal (Figure 3.9). Due to the limited supply of toxin available, thorough examination of possible additive effects of FTX and ω-agatoxin IVA could not be carried out. However a single experiment in which 1 mM sFTX and 10 nM ω-agatoxin IVA were combined provided no evidence to suggest that these two agents have an additive effect in blocking presynaptic Ca^{2+} flux (data not shown).

Additive Effects of ω-agatoxin IVA and ω-conotoxin MVIIC

In order to determine if the inhibitory effects of ω-conotoxin MVIIC and ω-agatoxin IVA were additive, both toxins were used in combination in a series of stopped-flow experiments. Buffers and dye-loaded synaptosomes were incubated with 300 nM ω-agatoxin IVA and a range of ω-conotoxin MVIIC concentrations from 10 nM to 1 μM. The Ca^{2+} flux was blocked to a greater degree in the presence of both 300 nM ω-agatoxin IVA and 1 μM ω-conotoxin MVIIC than in the presence of either toxin alone. Figure 3.10 summarizes the results from these experiments.

Effects of Other Pharmacological Agents on Presynaptic Ca^{2+} Signals

Aminoglycoside antibiotics: The aminoglycoside antibiotics tested in this preparation had few effects on presynaptic Ca^{2+} influx. Bekanomycin, gentamycin and streptomycin had no systematic effects at concentrations ranging from nanomolar to millimolar, but neomycin demonstrated some concentration-dependent block of the fluorescence signal (Figure 3.11).

Neurosteriods: Several neuroactive or neuroactive-like steroids were tested in stopped-flow fluorimetric experiments in order to determine if these agents have effects on presynaptic calcium channels. The four steroids that were tested were 5α-pregnane-3β,21-diol-20-one,5α-pregnan-3α-ol-20-one,5β-pregnane-3α,21-diol-20-one,5-pregnen-3β-ol,20-one sulphate, but none was found to have any systematic effect on presynaptic Ca^{2+} flux (Figure 3.12).

Modulation of Ca^{2+} flux through actions on presynaptic receptors: The activity of many voltage-sensitive calcium channels may be modulated directly by G-proteins or through a variety of second messenger pathways (Miller & Fox, 1990; Brown, 1993). Since presynaptic calcium channels are critical in mediating neurotransmitter release, detailed and subtle regulation of their function by G-protein linked second messenger systems might be expected. In order to evaluate possible modulation of Ca^{2+} flux by actions on presynaptic receptors, a series of studies using neuropeptide Y, baclofen and 2-chloroadenosine was carried out.

Neuropeptide Y or baclofen was preincubated with the dye-loaded synaptosomes for 5 or 10 minutes and depolarization was induced by mixing with either 25 or 50 mM KCl. Neither agent was shown to effectively modulate presynaptic Ca^{2+} influx (data not shown). Somewhat more extensive studies were carried out with 2-chloroadenosine. Initial results indicated a slight concentration dependent block of presynaptic Ca^{2+} flux with an IC_{50} of about 30nM when 2-chloroadenosine was incubated with synaptosomes

for at least 20 minutes prior to mixing (Figure 3.13). Additional experiments with shorter incubation times are also shown in Figure 3.13.

A summary of these pharmacological effects on presynaptic Ca^{2+} influx is presented in Table 3.1.

Table 3.1

The Effects of Pharmacological Agents on Presynaptic Ca^{2+} Influx Induced by 27.5 mM KCl Depolarization

LIGAND	%	IC_{50}	[MAX] tested
DIHYDROPYRIDINES			
nitrendipine	N/E		10μM
nimodipine	15%	100nM	10μM
BayK 8644	N/E		3μM
Verapamil	N/E		
ω-CONOTOXIN GVIA	15%	< 100nM	10μM
***AGELENOPSIS APERTA* VENOM**			
crude venom	50%		300ng/mL
ω-AGATOXIN IVA	30%	30nM	1μM
FTX	40%	4nL/mL	300nL/mL
synthetic FTX	50%		1mM
ω-CONOTOXIN MVIIC	40%	58nM	300nM
AMINOGLYCOSIDE ANTIBIOTICS			
neomycin	10%		1mM
streptomycin	N/E		1mM
kanamycin	N/E		3mM
gentamycin	N/E		1mM
NEUROACTIVE STEROIDS			
5α-pregnane-3β,21-diol-20-one	N/E		10μM
5α-pregnan-3α-ol-20-one	N/E		10μM
5β-pregnane-3α,21-diol-20-one	N/E		10μM
5-pregnen-aβ-ol,20-one sulphate	N/E		10μM
Neuropeptide Y	N/E		1μM
Baclofen	N/E		10μM
2-Chloradenosine	15%	30 nM	300 nM

N/E: no effect

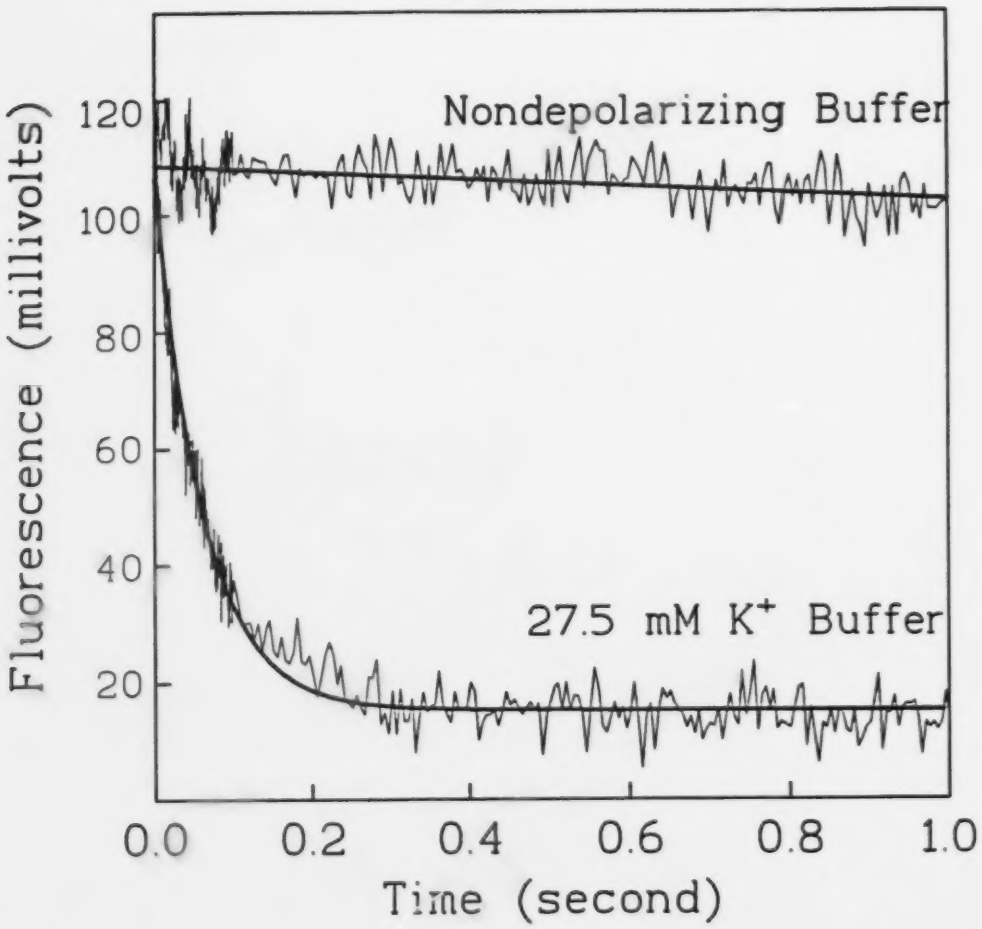


Figure 3.1 Effect of depolarization on changes in intrasynaptosomal Ca^{2+} concentration monitored by the fluorescence of Fura-2. The figure shows representative stopped-flow traces obtained upon rapid mixing of Fura-2 loaded synaptosomes with nondepolarizing (5 mM K^+) buffer and depolarizing buffer containing 50 mM K^+. All buffers were osmotically balanced and prepared using $choline^+$ rather than Na^+ to minimize Ca^{2+} flux due to Na^+/Ca^{2+} exchange. The quench observed with depolarization has been fitted by a single exponential equation as described in the text giving k_1 = 16.4 sec^{-1} and Amp_1 = 91.1 mv.

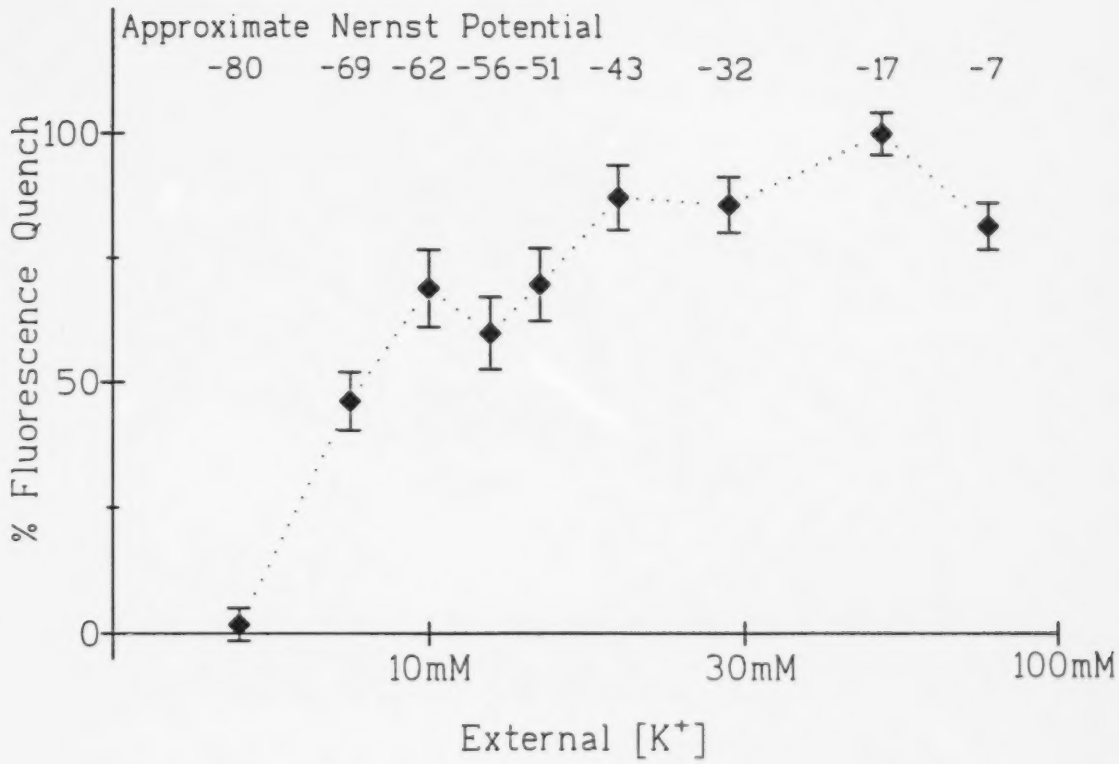


Figure 3.2 Effect of external K^+ concentration on the magnitude of the Fura-2 quench. Stopped-flow experiments were carried out using a series of buffers with elevated K^+ concentrations. The magnitude of the fluorescence quench increased as the K^+ concentration in the depolarizing buffer increased. Minor fluorescence changes occurring under nondepolarizing conditions ($[K^+]_{final}$=5mM) were subtracted from all traces. Maximum quench occurred with a $[K^+]_{final}$ of 52.5 mM. The approximate Nernst potentials indicated were calculated according to the K^+ equilibrium equation, assuming a synaptosomal resting potential of -80 mV (McMahon & Nicholls, 1991). Data represent the mean and SEM of six separate experiments.

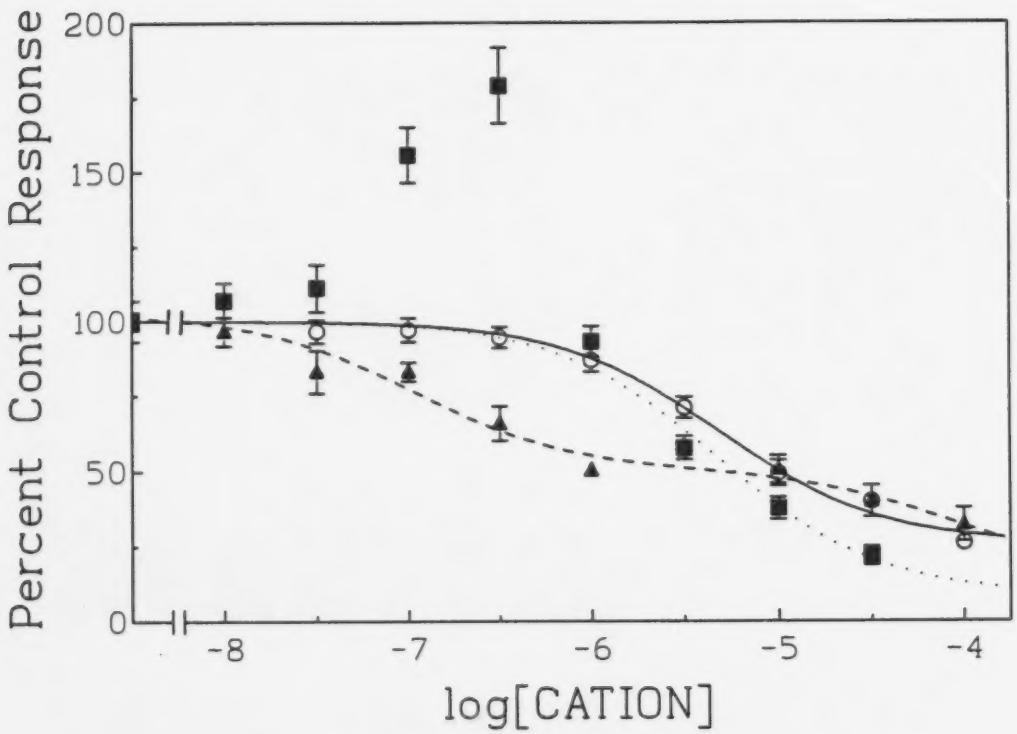


Figure 3.3 Block of depolarization-dependent changes in Fura-2 fluorescence by inorganic cations. Cd^{2+}(■), Co^{2+}(○), or La^{3+}(▲) was added to Fura-2 loaded synaptosomes and mixing buffers at least 15 minutes prior to stopped-flow experiments. The effects of Co^{2+} and Cd^{2+} (excluding high nanomolar concentrations of Cd^{2+}) were fit using a simple competitive model giving IC_{50} values of 4.7 μM and 4.8 μM respectively. The La^{3+} data were better described by a two component model with IC_{50} values of 133 nM and approximately 130 μM. Data represent the mean and SEM of five (Cd^{2+}), four (Co^{2+}), or seven (La^{3+}) separate experiments.

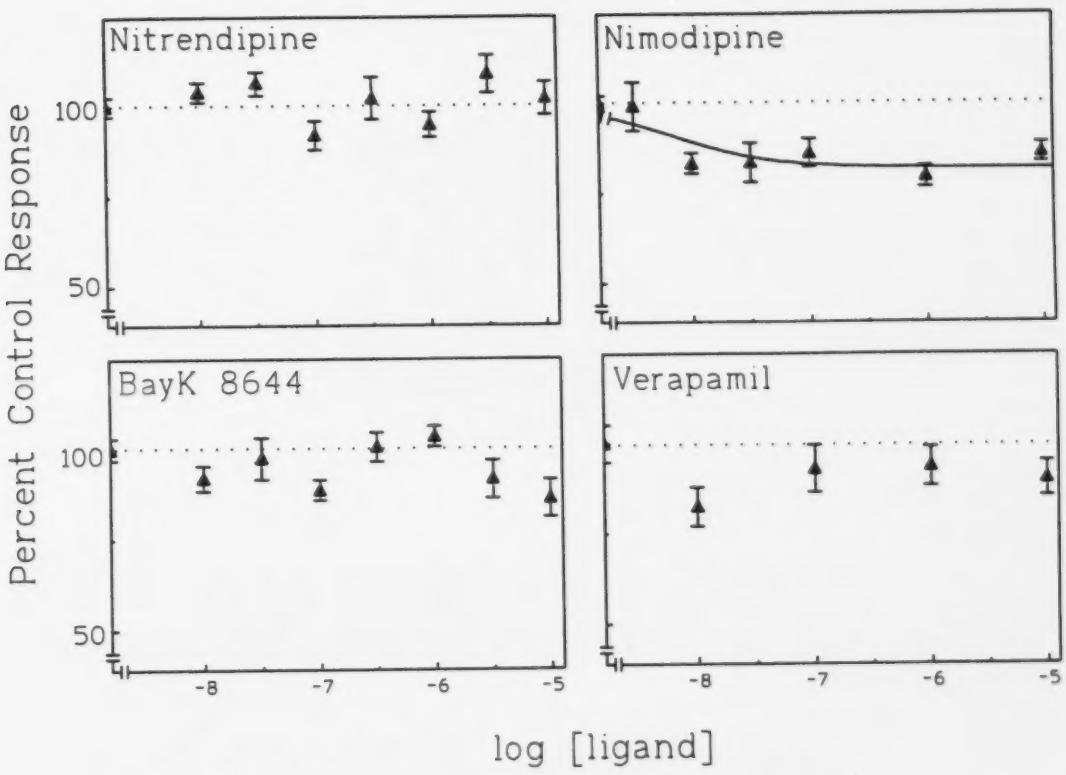


Figure 3.4 Effects of L-type calcium channel ligands, nitrendipine (A), nimodipine (B), Bay K8644 (C) and verapamil (D) on changes in Fura-2 fluorescence. Drugs were incubated with Fura-2 loaded synaptosomes and added to mixing buffers at least 15 minutes prior to stopped-flow fluorescence measurements. The amplitude of the depolarization-induced quench (27.5 mM $[K^+]_{final}$) at each concentration was normalized to that of control experiments in the absence of added drug. Data represent the mean and SEM of six (nitrendipine), eight (nimodipine), five (Bay K8644) and three (verapamil) separate experiments.

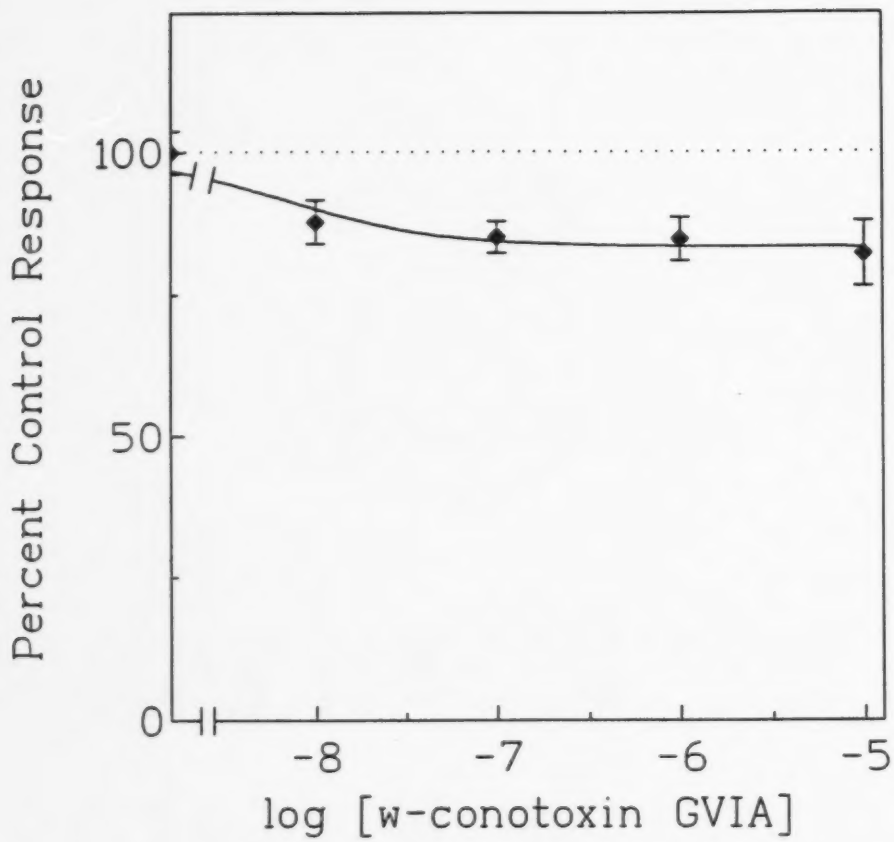


Figure 3.5 Effects of the N-type calcium channel antagonist ω-conotoxin GVIA on changes in Fura-2 fluorescence. The toxin was incubated with Fura-2 loaded synaptosomes and added to mixing buffers at least 20 minutes prior to stopped-flow fluorescence measurements. The amplitude of the depolarization-induced quench (27.5 mM $[K^+]_{final}$) at each concentration was normalized to that of control experiments in the absence of added toxin. ω-conotoxin GVIA inhibited the Ca^{2+} influx with an estimated IC_{50} equal to 66.5 nM. Data represent the mean and SEM of four to eight separate traces.

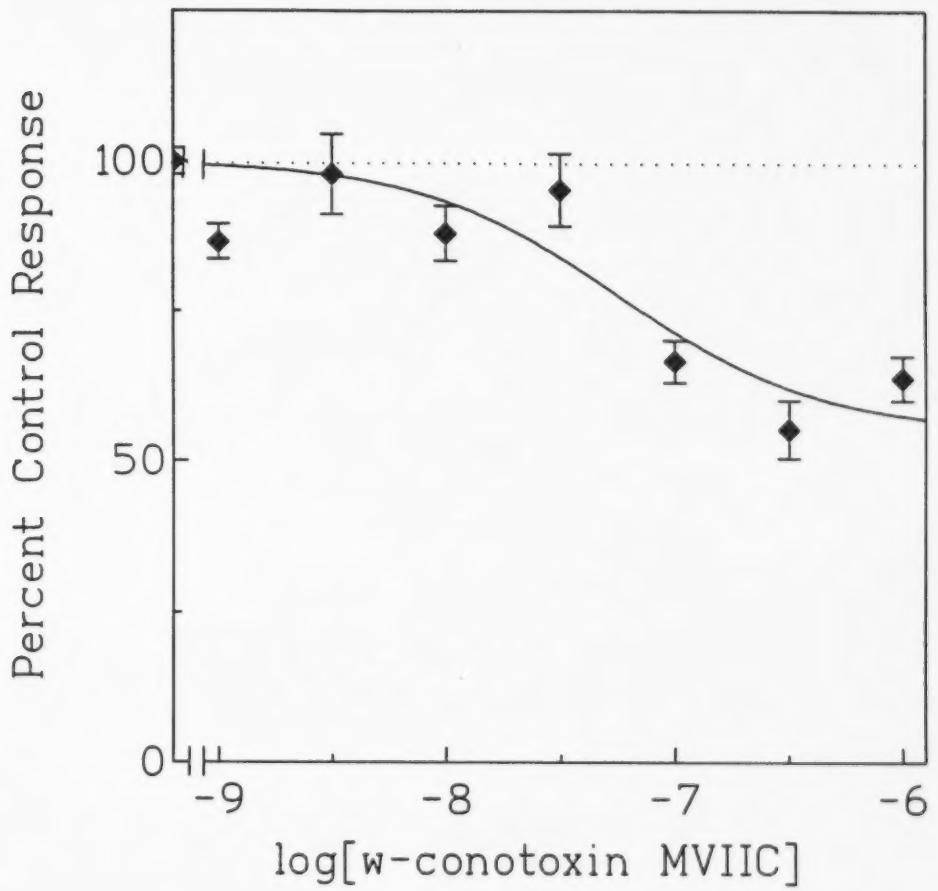


Figure 3.6 Effects of the Q-type calcium channel antagonist ω-conotoxin MVIIC on changes in Fura-2 fluorescence. The toxin was incubated with Fura-2 loaded synaptosomes and added to mixing buffers at least 20 minutes prior to stopped-flow fluorescence measurements. The amplitude of the depolarization-induced quench (27.5 mM $[K^+]_{final}$) at each concentration was normalized to that of control experiments in the absence of added toxin. ω-conotoxin MVIIC inhibited the Ca^{2+} influx with an estimated IC_{50} equal to 52 nM. Data represent the mean and SEM of seven separate experiments.

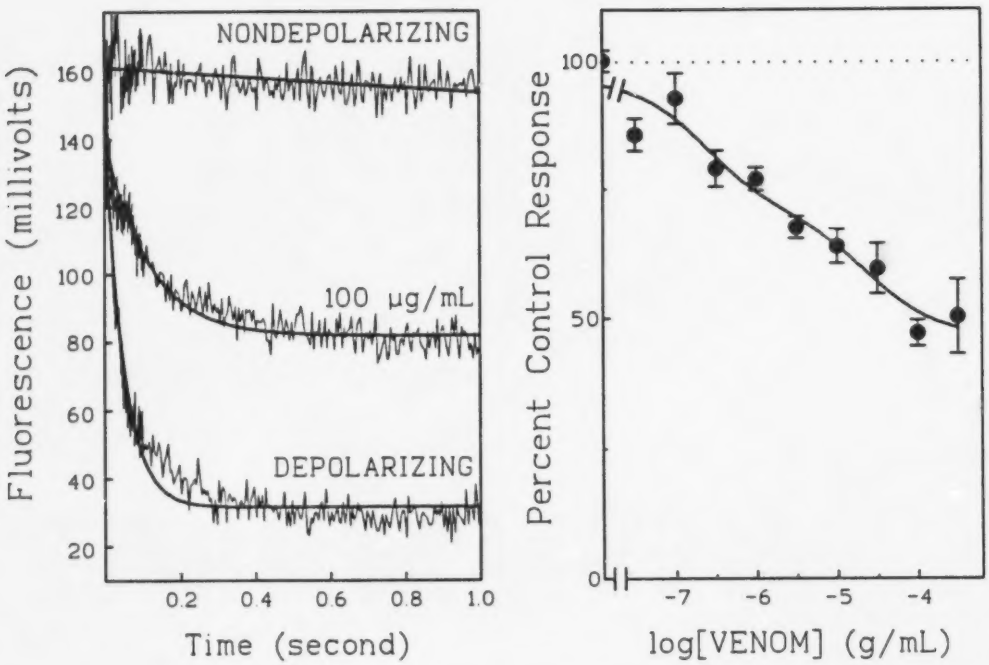


Figure 3.7 Inhibition of depolarization-induced changes in Fura 2 fluorescence by venom of *Agelenopsis aperta*. The *left* panel shows representative stopped-flow traces of the effect of increasing concentrations (0-100 µg/mL) of crude *Agelenopsis aperta* venom on the magnitude of the depolarization-induced ($[K^+]_{final}$=27.5 mM) quench of Fura-2 fluorescence. The *right* panel shows the effects of venom concentration on the magnitude of the quench normalized to control traces in the absence of venom (mean and SEM of five separate experiments). A two component model gave IC_{50} values of 107ng/mL and 15.8µg/mL.

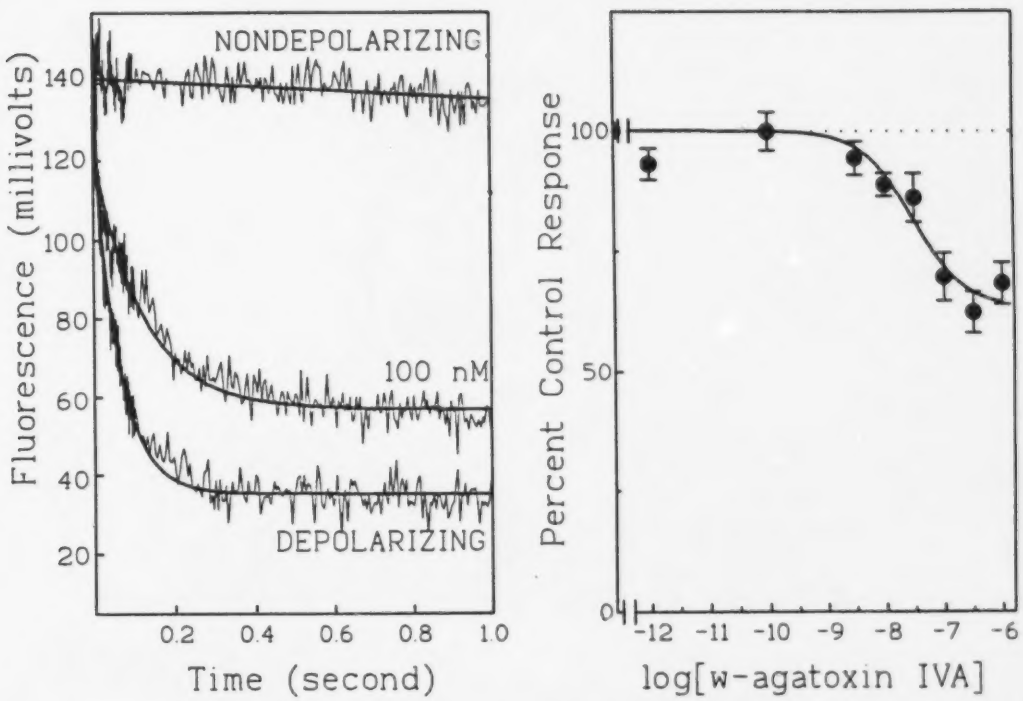


Figure 3.8 Effects of the P-type calcium channel antagonist ω-agatoxin IVA on depolarization-induced changes in Fura 2 fluorescence. The *left* panel shows representative stopped-flow traces of the depolarization-induced quench of Fura-2 fluorescence from one experiment with ω-agatoxin IVA. The *right* panel shows the effects of ω-agatoxin IVA (0 -1 μM) on the magnitude of the quench normalized to control traces in the absence of toxin (mean and SEM of traces from nine separate experiments). The IC_{50} value was estimated to be 30 nM. Data using purified and synthetic ω-agatoxin IVA were not significantly different and the figure shows pooled data using both toxin preparations.

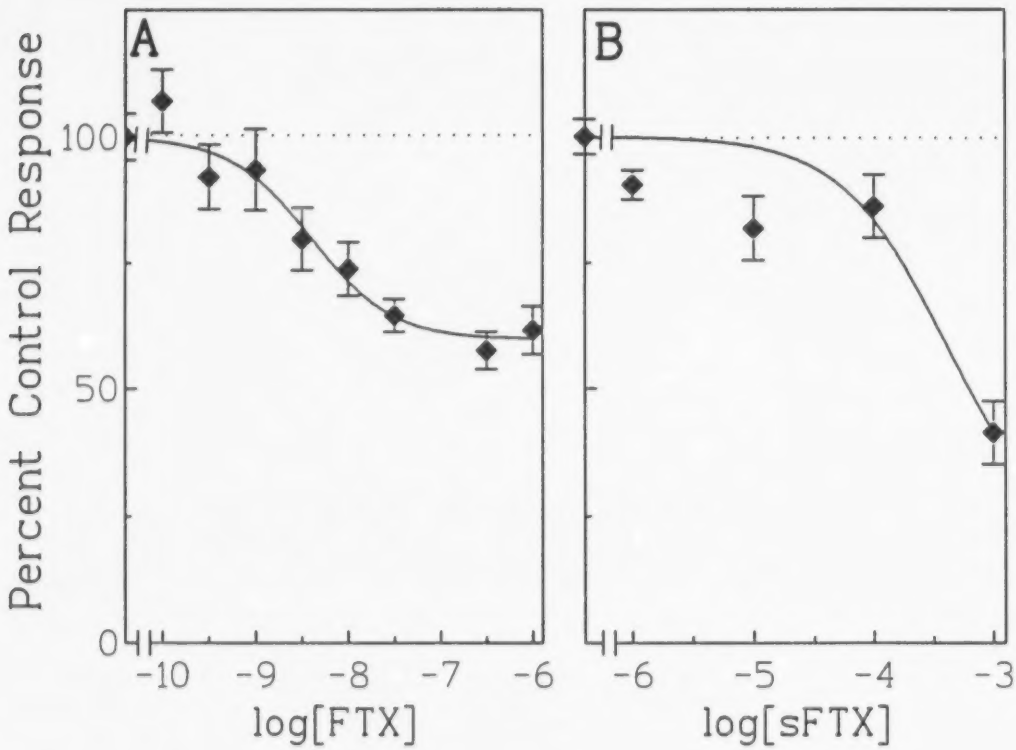


Figure 3.9 Inhibition of depolarization-induced changes in Fura-2 fluorescence by FTX and sFTX. Panel A illustrates the effects of purified FTX concentration (0-1μL/mL) on the magnitude of the quench normalized to control traces in the absence of toxin (mean and SEM of two separate experiments). An IC_{50} value was estimated to be 4nL/mL. Panel B shows the combined results of four separate stopped-flow experiments with synthetic FTX.

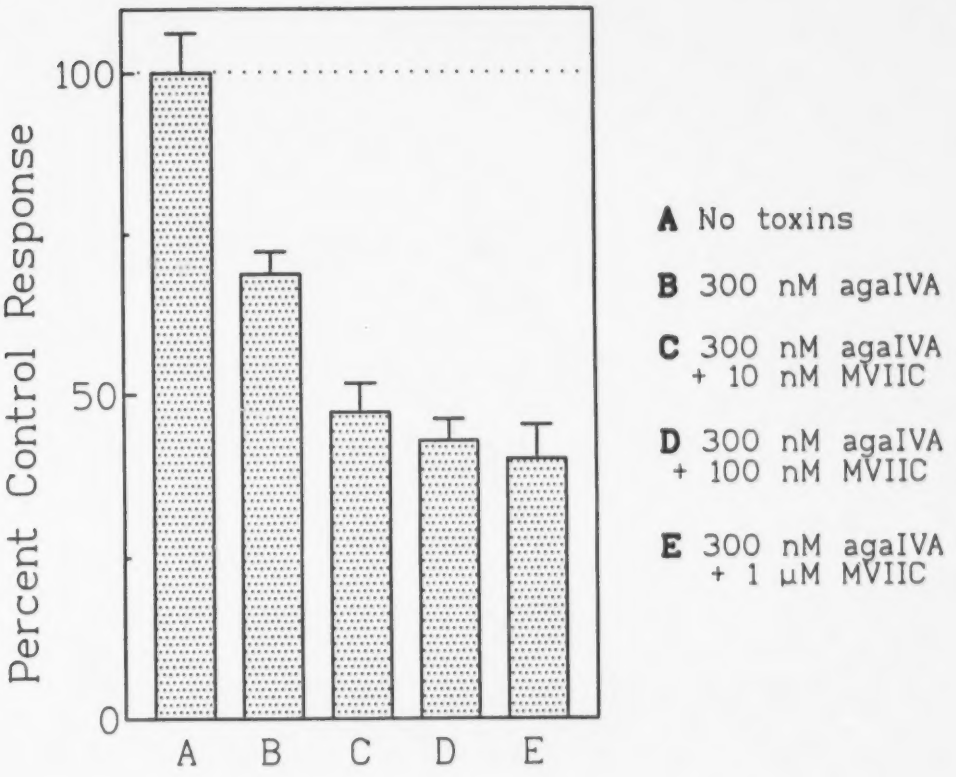


Figure 3.10 Additive effects of the P-type and Q-type calcium channel antagonists ω-agatoxin IVA and ω-conotoxin MVIIC on depolarization-induced changes in Fura 2 fluorescence. The toxins were incubated with Fura-2 loaded synaptosomes and added to mixing buffers at least 20 minutes prior to stopped-flow fluorescence measurements. The panel shows the overall results of two experiments (6-15 individual traces). Addition of 300 nM ω-agatoxin IVA blocks a significant portion of the Ca^{2+} signal and in combination with 10 nM to 1 μM ω-conotoxin MVIIC an additional component of the signal is inhibited. From left to right the bars indicated the percent of the baseline response measured with no toxin (A), 300 nM ω-agatoxin IVA (B), 300 nM ω-agatoxin IVA + 10 nM ω-conotoxin MVIIC (C), 300 nM ω-agatoxin IVA + 100 nM ω-conotoxin MVIIC (D), and 300 nM ω-agatoxin IVA + 1 μM ω-conotoxin MVIIC (E).

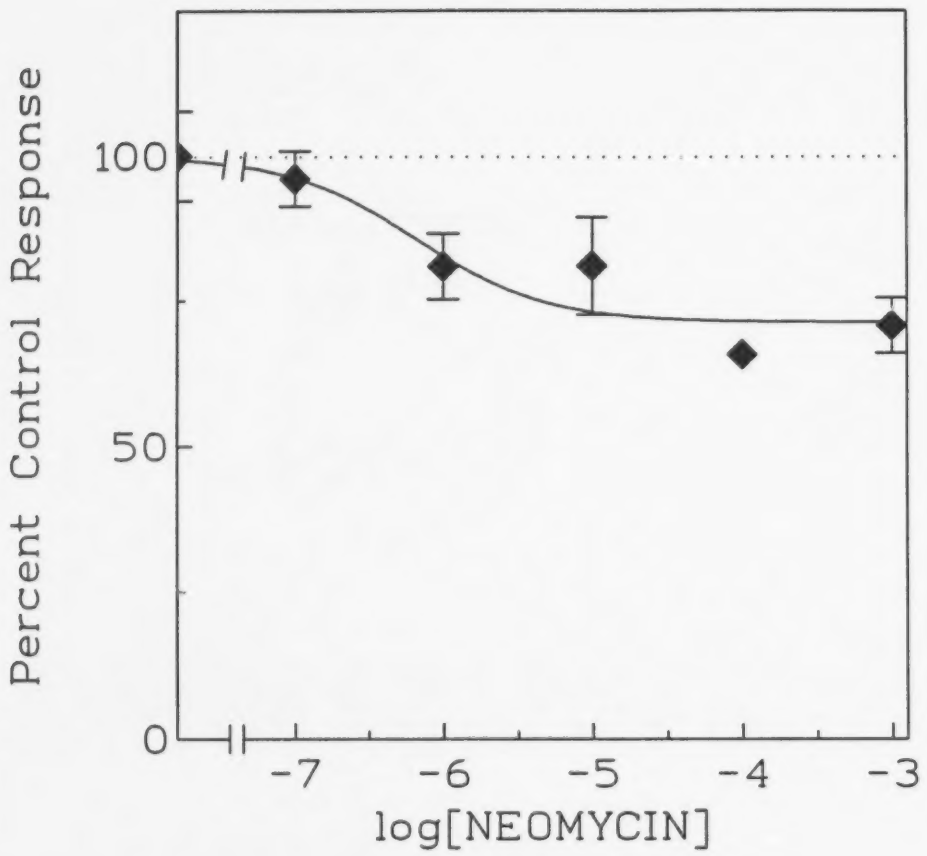


Figure 3.11 Effects of the aminoglycoside antibiotic, neomycin, on depolarization induced Ca^{2+} influx. Neomycin was incubated with Fura-2 loaded synaptosomes and mixing buffers at least 15 minutes prior to stopped-flow fluorescence measurements. The amplitude of the depolarization-induced quench (27.5 mM $[K^+]_{final}$) at each concentration was normalized to that of control experiments in the absence of added drugs. Data represent the mean and SEM of 5-8 separate traces.

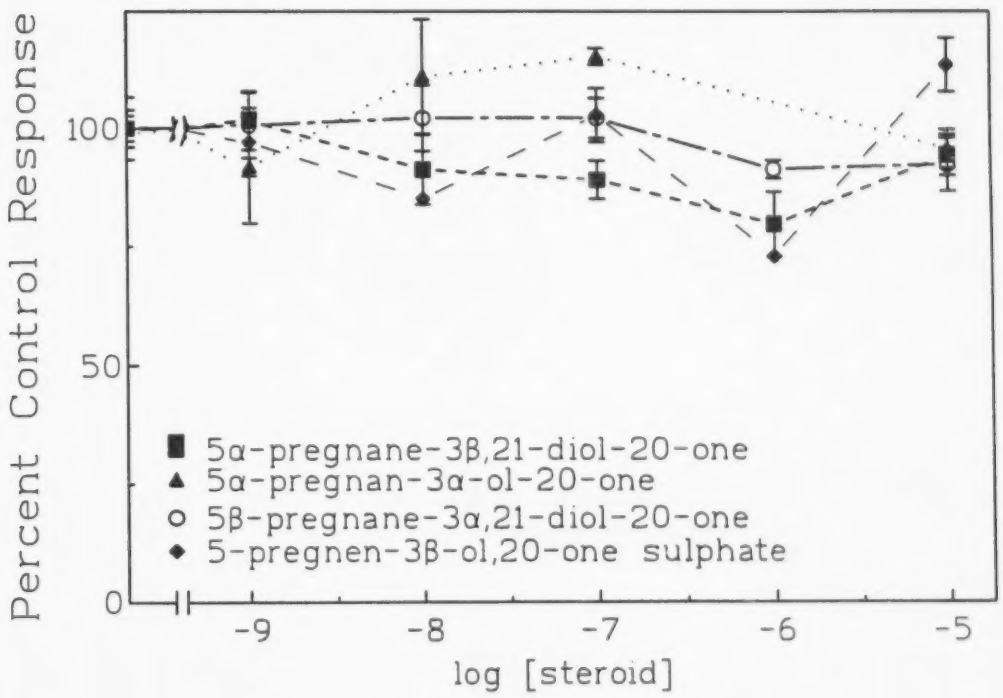


Figure 3.12 Neurosteriod effects on presynaptic Ca^{2+} flux. The drugs were incubated with Fura-2 loaded synaptosomes and mixing buffers at least 15 minutes prior to stopped-flow fluorescence measurements. The amplitude of the depolarization-induced quench (27.5 mM $[K^+]_{final}$) at each concentration was normalized to that of control experiments in the absence of added drugs. Data represent the mean and SEM of 3-9 (5α-pregnane-3β,21-diol-20-one), 3-5 (5α-pregnan-3α-ol-20-one), 7-20 (5β-pregnane-3α,21-diol-20-one), and 4-15 (5-pregnen-3β-ol,20-one sulphate) separate experiments.

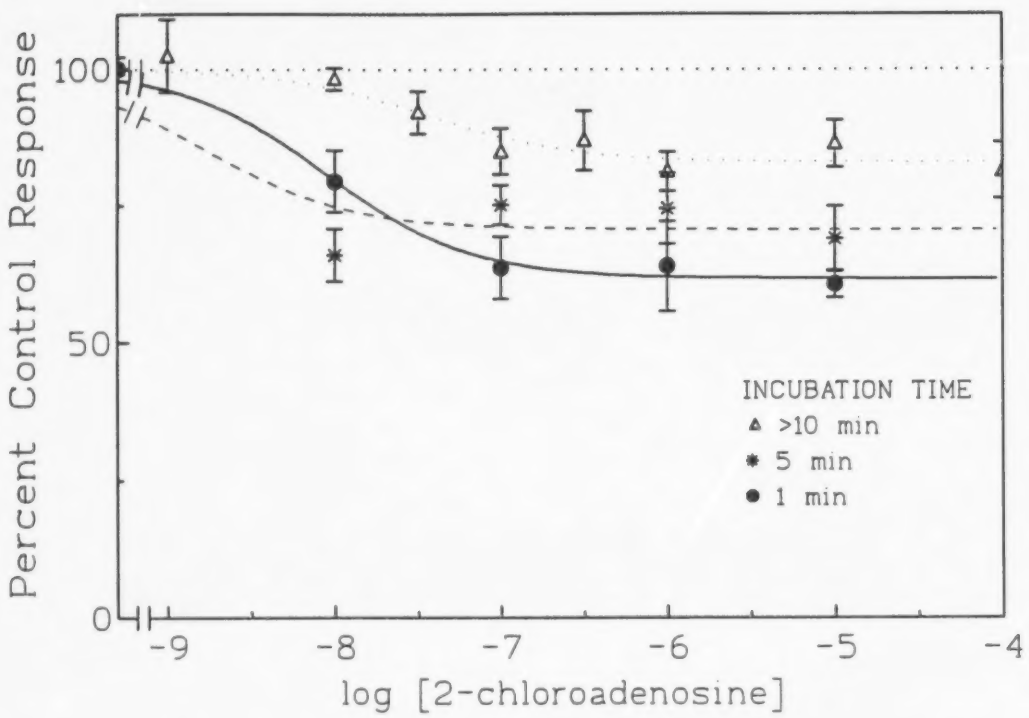


Figure 3.13 Effects of 2-chloroadenosine on changes in Fura-2 fluorescence. 2-Chloroadenosine was incubated with Fura-2 loaded synaptosomes and added to mixing buffers at least 15 minutes prior, or only one or five minutes prior to stopped-flow fluorescence measurements. The amplitude of the depolarization-induced quench (27.5 mM $[K^+]_{final}$) at each concentration was normalized to that of control experiments in the absence of added drug. Data represent the mean and SEM of 2-4 separate experiments.

DISCUSSION

The results reported above demonstrate that mammalian synaptosome preparations can be used to study rapid changes in nerve terminal Ca^{2+} signals resulting from membrane depolarization. By monitoring the fluorescence of Fura-2, changes in $[Ca^{2+}]_i$ can be measured on millisecond time scales, allowing detailed investigation of the effects of various calcium channel ligands. The K^+-dependence of the rise in $[Ca^{2+}]_i$ and the block by low concentrations of the inorganic calcium channel ligands, Cd^{2+}, Co^{2+} and La^{3+}, suggests that the major contributor to this process is the influx of Ca^{2+} through voltage-dependent calcium channels. The results of the experiments completed with this system, however, can not exclude a possible contribution to the observed fluorescence signal of Ca^{2+} released from intrasynaptosomal calcium stores. Thus although some portion of the Ca^{2+} signal can reasonably be assumed to arise from influx of Ca^{2+} through presynaptic calcium channels, based on the voltage-dependence, cation block and ligand sensitivity of the signal, it is recognized that the current methodology results in $[Ca^{2+}]$ changes that may not be entirely due to Ca^{2+} influx.

Stopped-flow experiments were carried out in buffers containing choline rather than Na^+ to minimize the possible contributions of Na^+-Ca^{2+} exchange mechanisms (Suszkiw *et.al.*,1986). Under these conditions, the depolarization-dependent changes in $[Ca^{2+}]_i$ are reasonably described by a single fast phase. Other studies of time-resolved changes in intracellular $[Ca^{2+}]$ using similar techniques report that in buffers containing Na^+, KCl-induced depolarization resulted in a biphasic rise in $[Ca^{2+}]_i$ (Lentzner *et.al.*, 1992; Bartschat & Rhodes, 1995). Lentzner *et.al.* (1992) concluded that the faster phase (which had a similar rate to that reported here) was mediated by voltage-dependent calcium channels since the slower component ($k \approx 1.5$ sec^{-1}) was greatly reduced in Na^+-depleted buffers and was likely, therefore, to be due to Na^+-Ca^{2+} exchange operating in reverse mode.

Neuronal calcium channels are sensitive to block by divalent and trivalent cations (Nachshen, 1984; Suszkiw *et.al.*, 1989). The results in Figure 3.3 show that La^{3+} inhibited about 70% of the response very potently (IC_{50} = 134 nM). The dose dependence of block by La^{3+} was, however, biphasic, suggesting the involvement of

different types of calcium channel or Ca^{2+} source. Cd^{2+} consistently potentiated the response at low concentrations and, at higher concentrations (1-100 μM) produced a complete block. Cd^{2+} may permeate L-type calcium channels or otherwise cross the membrane and binds with higher affinity than Ca^{2+} to Fura-2 (Sun & Benishin, 1989; Hinkle *et.al.*,1992). Ca^{2+} is displaced from a Fura 2-Ca^{2+} complex by Cd^{2+} and the fluorescence is enhanced (Hinkle *et.al.*, 1992). Thus the greater fluorescent signal obtained in the current study at submicromolar Cd^{2+} concentrations, might be explained by Cd^{2+} permeating the synaptosomes and binding to Fura-2 or displacing Ca^{2+} from external Fura 2-Ca^{2+} complexes, while at higher concentrations, the Cd^{2+} effectively blocks the Ca^{2+} influx that accounts for most of the fluorescence quench. Although there is currently no clear explanation for the potentiating effect, it is interesting to note that a similar phenomenon is also evident in a previous report (Lentzner *et.al.*, 1992). Co^{2+} also inhibited the flux response with an IC_{50} of about 5 μM, which is about two orders of magnitude more potent than previously reported (Suszkiw *et.al.*, 1989; Tareilus *et.al.*, 1993).

The dependence of the changes in Fura-2 fluorescence on the external K^+ concentration showed that the response was voltage-dependent and that the threshold for activation was at relatively negative membrane potentials, with a significant response occurring upon elevation of extrasynaptosomal K^+ from 5 mM to 10 mM (Figure 3.2). Breer and co-workers (Tareilus & Breer, 1992; Tareilus *et.al.*, 1993), who also used Na^+-depleted, choline-substituted media, observed a similar potential dependence although with a higher threshold for activation at about 15 mM K^+. In buffer containing Na^+, Lentzner *et.al.* (1992) observed that the fast phase which, as described above, is thought to be due to Ca^{2+} influx through voltage-dependent calcium channels, had a threshold for activation at about 10 mM external K^+ but did not appear to saturate at K^+ concentrations up to 100 mM. Taken together, these results demonstrate that depolarization-induced Ca^{2+} uptake by mammalian synaptosomes occurs at more negative potentials than expected for the "high voltage-activated" L- and N-type calcium channels (Miller & Fox, 1990) but the potential-dependence is similar to that reported for P-type

channels (Llinás *et.al.*, 1989) Although the magnitude of the observed fluorescence quench was dependent on the external K^+ concentration, the rate showed little such dependency. The estimated rate constants of 10 -15 sec^{-1} are similar to those reported previously (Lentzner *et.al.*, 1992; Bartschat & Rhodes, 1995), but are considerably slower than the timescales of physiological neurotransmitter release. This is not surprising since, in the present experiments, only changes in Ca^{2+} concentrations in the bulk cytosol can be determined, and not Ca^{2+} transients that occur at the active zones associated with neurotransmitter release. This may also explain the lack of voltage-dependence of the observed rates of the fluorescence quench since diffusion and buffering of the Ca^{2+}, rather than channel gating, is likely to be rate limiting.

The L-type calcium channel ligands tested were generally ineffective in modifying the presynaptic Ca^{2+} signals. There have been reports that, in presynaptic terminals, verapamil, a phenylalkyamine L-type channel antagonist, significantly blocks $^{45}Ca^{2+}$ uptake and changes in $[Ca^{2+}]_i$ measured with Fura-2 (Canzoniero *et.al.*, 1993; Sze & Iqbal, 1994); however this block appears to be primarily due to actions of verapamil on the Na^+/Ca^{2+} antiporter in the plasma membrane rather than effects on voltage-sensitive calcium channels (Sitges *et.al.*, 1990; Canzoniero *et.al.*, 1993). The lack of inhibition of Ca^{2+} flux by verapamil observed in the current study is consistent with a primary action on the Na^+/Ca^{2+} exchanger since, in the low Na^+ conditions used, the Na^+/Ca^{2+} exchange would not contribute significantly to the Ca^{2+} signals.

On rapid time scales, nitrendipine, even at micromolar concentrations, did not have any consistent blocking action although the related drug, nimodipine, did block 10-15% of the response. Bay K8644, the only agonist tested, was without a systematic effect. Previous reports in which Bay K8644 effects on $^{45}Ca^{2+}$ uptake into synaptosomes were measured have provided some evidence for an enhancement of uptake (White & Bradford, 1986) but other results have indicated a lack of effect (Suszkiw *et.al.*, 1989). In other studies of L-type antagonists on neuronal calcium channels, few significant effects have been demonstrated (Lentzner *et.al.*, 1992; Lundy *et.al.*, 1991; Perrier *et.al.*, 1992; Regan *et.al.*, 1991; Suszkiw *et.al.*, 1989; Tareilus *et.al.*, 1993; Sze & Iqbal,

1994; Maubecin *et.al.*, 1995). Nimodipine, however, has previously been shown to have some selective activity on neuronal tissues (McCarthy & TanPiengo, 1992; Scriabine *et.al.*, 1989; Fanelli *et.al.*, 1994). Overall it appears that dihydropyridine-sensitive voltage-dependent calcium channels are not the major channels involved in voltage-dependent Ca^{2+} uptake by mammalian nerve terminals.

It has previously been suggested that L-type channels may be more abundant in the neuronal soma where they may regulate events such as changes in gene expression, whereas N-, P- and/or Q-type channels may be important in the nerve terminals for the regulation of neurotransmitter release (Ahlijanian *et.al.*, 1990; Sher *et.al.*, 1991; Bertlino & Llinás, 1992; Olivera *et.al.*, 1995). Generally ω-conotoxin GVIA has been shown to be minimally involved in blocking mammalian presynaptic Ca^{2+} fluxes (Regan *et.al.*, 1991; Lundy *et.al.*, 1991; Suszkiw *et.al.*, 1989; Perrier *et.al.*, 1992; Hillyard *et.al.*, 1992; Pocock & Nicholls, 1992; Maubecin *et.al.*, 1995), although strong evidence for a more prominent role of ω-conotoxin GVIA-sensitive calcium channels in avian (Scheer, 1990; Lundy *et.al.*, 1991; Maubecin *et.al.*, 1995) or other species (Suszkiw *et.al.*, 1987) has been presented. In agreement with previous reports (Lundy *et.al.*, 1991; Suszkiw *et.al.*, 1989; Tareilus *et.al.*, 1993; Maubecin *et.al.*, 1995), it was found that the voltage-dependent calcium channels in rat brain synaptosomes are relatively insensitive to this toxin (Figure 3.5). Lentzner *et al.* (1992) found that the effects of ω-conotoxin GVIA were erratic but suggested that, since this toxin sometimes blocked Ca^{2+} uptake, N-type channels may be involved. In the present study small, though quite consistent effects were observed with this toxin. Thus some involvement of N-type calcium channels in rat brain synaptosome preparations cannot be excluded.

Submicromolar concentrations of ω-conotoxin MVIIC blocked 40% of the presynaptic Ca^{2+} flux. This peptide toxin, purified from the fish-hunting snail *Conus magus*, was recently identified (Hillyard *et.al.*, 1992) and has subsequently been evaluated in several preparations (Zhang *et.al.*, 1993; Robichaud *et.al.*, 1994; Turner *et.al.*, 1995; Wu & Saggau, 1995). The calcium channels or currents that this toxin blocks have been designated as "Q" (Zhang *et.al.*, 1993; Wheeler *et.al.*, 1994) although

there is significant debate as to the particular specificity of this toxin (Dunlap *et.al.*, 1994; Turner *et.al.*, 1995; Wu & Saggau, 1995). At mammalian synapses, ω-conotoxin MVIIC blocks a component of Ca^{2+} flux insensitive to block by other calcium channel antagonists (Hillyard *et.al.*, 1992; Wheeler *et.al.*, 1994), although the combination of antagonists at L-, N-, P- and Q-type calcium channels has not been shown to block all neuronal Ca^{2+} currents (Zhang *et.al.*, 1993; Turner *et.al.*, 1995; Wu & Saggau, 1995). In the current studies, ω-conotoxin MVIIC was very effective in blocking a significant portion of the presynaptic Ca^{2+} influx.

Depolarization-induced Ca^{2+} uptake was inhibited by about 50% in the presence of 10-100 μg/mL crude venom from the funnel web spider, *Agelenopsis aperta* (Figure 3.7). The results with FTX, a polyamine purified from *Agelenopsis aperta*, indicate that this component of the spider venom inhibits 40% of presynaptic Ca^{2+} influx in synaptosomes at submicrolitre/mL concentrations (Figure 3.9). Recently Uchitel and co-workers, using a $^{45}Ca^{2+}$ flux technique, reported 0.7 μL/mL FTX completely blocked the depolarization dependent uptake of Ca^{2+} by synaptosomes (Uchitel *et.al.*, 1992). In the same study, the synthetic FTX (sFTX) polyamine was also shown to be effective in blocking Ca^{2+} influx at low millimolar concentrations. The limited results obtained with sFTX in the current study concur with millimolar concentrations being required for an effect to be observed (Figure 3.9). The difference in potency observed with FTX and sFTX probably reflects differences in the molecular structure of the two compounds, since the actual structure of FTX is unknown (Cherksey *et.al.*, 1991). It is possible that the purified FTX may also contain some peptide toxins which would contribute to the activity observed; however extensive analysis of the purified compound suggests peptide contamination is minimal (Llinás *et.al.*, 1992).

The peptide toxin from *Agelenopsis aperta*, ω-agatoxin IVA, also potently blocked a component (30%) of Ca^{2+} influx into synaptosomes (Figure 3.8). Breer and co-workers, using a stopped-flow system and rat cortical synaptosomes loaded with Indo-1 or Fluo-3, have reported a similar block (46%) of the high potassium induced signal with 100 nM ω-agatoxin IVA (Tareilus *et.al.*, 1993). A similar potency, but greater inhibition

to 70-85% of $^{45}Ca^{2+}$ influx in synaptosomes has been observed with ω-agatoxin IVA (Mintz *et.al.*, 1992; Maubecin *et.al.*, 1995). Other peptide toxins purified from funnel web spider venoms have also been shown to partially block $^{45}Ca^{2+}$ uptake (Olivera *et.al.*, 1991; Lundy *et.al.*, 1991; Pocock *et.al.*, 1992; Venema *et.al.*, 1992) or depolarization-dependent changes in the fluorescence of Ca^{2+}-sensitive dyes in mammalian synaptosomes (Pocock & Nicholls, 1992; Maubecin *et.al.*, 1995).

Although only limited experiments were carried out using both ω-conotoxin MVIIC and ω-agatoxin IVA, the results indicate that the two toxins inhibit more of the presynaptic Ca^{2+} influx when used in combination than when acting alone (Figure 3.10). In electrophysiological studies, both whole cell currents in cultured granule cells (Wheeler *et.al.*, 1994) or *in vitro* recordings from the hippocampal slice (Wu & Saggau, 1995) have demonstrated distinct components of Ca^{2+} current that are blocked by ω-conotoxin MVIIC or ω-agatoxin IVA, although both toxins apparently affect some Ca^{2+} current in common. Turner *et.al.* (1995) found that ω-conotoxin MVIIC blocked radiolabelled transmitter release from rat cortical synaptosomes to the same degree as ω-conotoxin GVIA and ω-agatoxin IVA in combination. The present results are consistent with some overlap of the calcium channel specificity of ω-conotoxin MVIIC and ω-agatoxin IVA.

The data from these studies using subtype specific channel ligands, suggest that K^+-induced presynaptic Ca^{2+} influx is primarily mediated by channels sensitive to P and Q-type antagonists.

A number of additional drugs were tested for modulatory effects on synaptosomal calcium channels using the stopped-flow fluorescence technique. Most aminoglycoside antibiotics studied did not affect depolarization-induced Ca^{2+} influx measured in any consistent manner. A presynaptic action of aminoglycoside antibiotics has been postulated from clinical observations that a side effect of these agents is neuromuscular block (Atchison *et.al.*,1988). Demonstration that neomycin inhibited [^{125}I]ω-conotoxin GVIA binding to synaptic membranes (Knaus *et.al.*, 1987), suggested that the mechanism of block may be via a presynaptic calcium channel. Of the four drugs tested, neomycin

was the only agent that demonstrated a dose-dependent effect at submillimolar concentrations, inhibiting approximately 25% of the observed flux with an IC_{50} of 10-100 μM (Figure 3.11). This is in reasonable agreement with a recent report in which 300 μM neomycin reduced synaptosomal $^{45}Ca^{2+}$ uptake by approximately 30%, as well as changes in $[Ca^{2+}]_i$ measured with Fura-2 following potassium-induced depolarization (Canzoniero *et.al.*,1993). In studies comparing a number of aminoglycoside antibiotics, neomycin was shown to most effectively block both depolarization-induced Ca^{2+} entry into immature rat slices with an IC_{50} of about 700 μM (Perrier *et.al.*,1992) and $^{45}Ca^{2+}$ uptake in synaptosomes with a potency of about 10 μM (Atchison *et.al.*,1988). Although neomycin may block a component of presynaptic Ca^{2+} flux through voltage-sensitive calcium channels (Canzoniero *et.al.*,1993), the relatively low affinity of this drug limits its usefulness for further pharmacological characterization of presynaptic calcium channels.

Recently there has been an increased interest in the mechanism of steroid action in the central nervous system. Specific steroid binding sites have been identified in the plasma membrane of neural cells but the signal transduction mechanism following receptor activation is unclear (Duval *et.al.*, 1983). The present results for the four steroids tested suggest that under the experimental conditions used, they do not modulate presynaptic calcium influx through voltage-sensitive channels. Pregnenalone and related neurosteroids have been reported to block Ca^{2+} currents in dissociated hippocampal neurons, whereas progesterone was without effect (Spence *et al*, 1991; ffrench-Mullen & Spence, 1991). A recent report in which a number of glucocorticoids and gonadal steroids were incubated with rat cortical synaptsomes prior to measurement of $^{45}Ca^{2+}$ uptake was stimulated by either high potassium or veratridine, showed that the glucocorticoids enhanced $^{45}Ca^{2+}$ uptake while the other steroids were without effect (Sze & Iqbal, 1994). These authors propose that the increased Ca^{2+} flux induced by the steroid is due to a calmodulin-dependent modulation of presynaptic calcium channels. It is possible that additional stopped-flow studies utilizing different steroid incubation periods, temperatures or other experimental conditions would permit further dissection

of possible neurosteroid effects at the presynaptic terminal.

The membrane of the nerve terminal contains many types of presynaptic receptors. Regulation of Ca^{2+} flux through voltage-sensitive calcium channels by second messenger systems has been directly demonstrated in several tissues (Fox *et.al.*, 1991; Scott *et.al.*, 1991). Direct measurements of similar actions occurring in the presynaptic terminal are difficult due to the small size and relative inaccessiblity of these areas of the neuron; however, stopped-flow fluorimetry provides a means to more directly measure changes in presynaptic Ca^{2+} concentrations. A series of experiments were carried out to test for modulatory effects on depolarization-induced Ca^{2+} influx following preincubation with neuropeptide Y (active at presynaptic Y_2 receptors), baclofen (a $GABA_B$ receptor agonist) or 2-chloroadenosine (an agonist at A_1-type adenosine receptors). In these experiments NPY and baclofen were without effect.

Recently two reports have been published in which the effects of presynaptic receptor stimulation or direct second messenger activation on time-resolved changes in intrasynaptosomal Ca^{2+} were monitored (Tareilus *et.al.*, 1994; Bartschat & Rhodes, 1995). In stopped-flow experiments in which $GABA_B$ receptor activity was studied, Breer and co-workers (Tareilus *et.al.*, 1994), found that the period between presentation of 100 μM baclofen and K^+-depolarization of hippocampal synaptosomes was critical. The Ca^{2+} response was maximally attenuated when baclofen was presented for 250 milliseconds while no inhibition was evident if the period was increased to 500 milliseconds. In addition, these workers found that $GABA_B$ receptor agonists were effective in inhibiting Ca^{2+} flux in hippocampal synaptosomes only. In the current study, NPY or baclofen was permitted to equilibrate with cortical synaptosomes for 10 minutes prior to mixing or just 5 minutes in some experiments with NPY. Also in tests with NPY, a lower level of depolarization (15 mM KCl) failed to affect Ca^{2+} influx. The different experimental conditions, as well as the use of cortical rather than hippocampal synaptosomes may well account for the differences in the results obtained in the current study. Bartschat and Rhodes (1995), using hippocampal synaptosomes, modified the phosphorylation state of the preparation directly, either by activating protein kinase C with a phorbol ester, or by inhibiting phosphatase with okadaic acid. Increased

phosphorylation levels led to greater Ca^{2+} influx following high potassium depolarization. The phorbol ester or okadaic acid was incubated with dye-loaded synaptosomes for 30 minutes prior to stopped-flow mixing. These two reports of presynaptic modulation of calcium channel function illustrate some of the complexities involved in studying second messenger systems.

More detailed experiments were completed with 2-chloroadenosine. Although 2-chloroadenosine inhibited about 10% of the Ca^{2+} influx when the standard incubation conditions were employed, 25-30% of the Ca^{2+} signal was inhibited when the 2-chloroadenosine was permitted to incubate with the synaptosomes for less than 1 minute prior to mixing of the vesicles and buffers in the stopped-flow apparatus (Figure 3.13). The greater modulatory effect observed with a briefer exposure to 2-chloroadenosine accords with the $GABA_B$ results of Breer *et.al.* discussed above, although in their study, only subsecond exposure to the presynaptic agonist was permitted (Tarielus *et.al.*, 1994b). Whether the effect on the presynaptic calcium channel is due to a diffusible second messenger or a direct action of the G-protein on the channel, functional modulation could be expected to occur quite rapidly. Prolonged exposure to the presynaptic agonist may lead to desensitization of the response and compensation by other cellular mechanisms such that no change is detectable. In addition, the nerve terminals used have been frozen and it is possible that some intracellular components critical for mediating a second messenger system may be impaired in a thawed versus fresh synaptosome prepartion.

The pharmacological results presented here indicate that the primary voltage-sensitive Ca^{2+} influx in rat cortical nerve terminals is through calcium channels sensitive to block by P and Q-type antagonists, particularly ω-agatoxin IVA, FTX and ω-conotoxin MVIIC. Modulation of channel function by several aminoglycoside antibiotics, neurosteroids, baclofen or NPY was not detectable with the experimental protocol employed, however altering the incubation and timing characteristics employed suggest stopped-flow fluorimetry will prove to be a useful technique in investigating second messenger modulation of presynaptic calcium channel activity.

Chapter Four

Investigation of the Activity of Voltage-Dependent Calcium Channels Following 15 mM KCl or 1 mM 4-Aminopyridine Depolarization

RESULTS

Since depolarization of the membrane potential is the trigger for Ca^{2+} flux through voltage-sensitive calcium channels, altering the degree of the depolarization or the method of inducing depolarization may influence the pharmacological characteristics of this flux. In order to investigate this possibility, Fura-2 loaded synaptosomes were depolarized by an intermediate level of KCl (15 mM) or by blocking potassium channels with 1 mM 4-AP. The results were compared with findings following 27.5 mM KCl stimulation (Chapter 3).

Membrane Potential Changes: Membrane potential changes were monitored by the fluorescence changes of the voltage sensitive dye $diSC_3(5)$. In the presence of an electrical potential across the synaptosomal membrane, this cationic dye associates with the membrane constituents and there is a consequent quench in the fluorescence. When the synaptosomes are depolarized, the dye dissociates from the membrane and the fluoresence signal is increased (Waggoner, 1979). Figure 4.1 shows a representative fluorimeter trace from one experiment. The least quench is observed with $[K^+]_o$ = 27.5 mM, while $[K^+]_o$ = 15 mM produced an intermediate level of fluorescence quench. There was typically no difference in the fluorescence observed when synaptosomes were mixed with nondepolarizing buffer or buffer containing 1 mM 4-AP, suggesting that the overall degree of depolarization induced by 4-AP is less than can be detected with this membrane potential dye. The membrane potential change may not be detectible due to either an overall low degree of depolarization or perhaps only a relatively small fraction of the synaptosomes are depolarized with 4-AP.

Stopped-Flow Fluorimetry: Figure 4.2 illustrates typical stopped-flow traces following

rapid mixing of Fura-2 loaded synaptosomes with an equal volume of the indicated buffer. Sodium buffers were used in 4-AP experiments due to the role of Na^+ influx in this depolarization method, while 15 mM KCl buffers contained choline chloride. The figure shows the relative change in fluorescence under each condition and the single exponential fit of the trace. As described in Chapter 3, the amplitude of the exponential fit is representative of the amount of Ca^{2+} influx that occurs following stimulation. The magnitude of the quench of Fura-2 fluorescence following depolarization by 1 mM 4-AP was less than the quench observed following elevation of external potassium to 15 mM or higher levels.

A series of experiments in which the dose-dependence of 4-AP induced fluorescence changes was monitored indicated that 1 mM 4-AP was the optimal concentration. A minimal quench was observed with submillimolar concentrations and, with 10 mM 4-AP, the reaction trace profile suggested there was considerable Ca^{2+} entry continuing after the initial 100 milliseconds (data not shown). Also such high concentrations of 4-AP may result in significant non-specific effects (Carvalho *et.al.*, 1995).

The 4-AP induced signals were dependent on Na^+ entry through sodium channels since 200nM TTX effectively blocked the response. TTX had no effect on KCl-induced fluorescence changes. The Ca^{2+} influx following 1 mM 4-AP stimulation was completely blocked by 10 μM Cd^{2+} consistent with Ca^{2+} entry through voltage-dependent calcium channels.

Pharmacology of Ca^{2+} Influx

L-type Ligands: Figure 4.3 shows the effects of nitrendipine and nimodipine on 15 mM KCl and 1mM 4-AP-induced Ca^{2+} influx. Nitrendipine did not modify the Ca^{2+} signal when the synaptosomes were depolarized with 15 mM KCl, but blocked about 20% of the 1 mM 4-AP induced Ca^{2+} influx with an estimated IC_{50} of 77.6 nM. Nimodipine did not inhibit 15 mM K^+-induced Ca^{2+} influx in any systematic way. 4-AP induced Ca^{2+} influx was, however, 25% blocked with an estimated IC_{50} of 63 nM.

Bay K8644, a dihydropyridine agonist, and the phenylalkylamine, verapamil were

ineffective in blocking Ca^{2+} entry into synaptosomes whether 15 mM KCl or 1 mM 4-AP was employed as the depolarizing stimulus (data not shown).

N-,P- and Q-type Ligands: Voltage-dependent modulation of the sensitivity of presynaptic Ca^{2+} influx by N-,Q- and P-type channel antagonists was evaluated by using ω-conotoxin GVIA, ω-conotoxin MVIIC and ω-agatoxin IVA. Each toxin was tested at 10 nM and 100 nM or 100 nM and 1 μM concentrations, based on the IC_{50} values obtained in the earlier studies with 27.5 mM KCl depolarization (Chapter Three).

Figure 4.4 shows the results of stopped-flow experiments with ω-conotoxin GVIA following depolarization with 15 mM KCl and 1 mM 4-AP. ω-conotoxin GVIA blocked approximately 10% the Ca^{2+} flux observed following stimulation by 15 mM KCl ($IC_{50} \approx$ 300 nM). With 4-AP depolarization, 100 nM and 1 μM ω-conotoxin GVIA were equally effective in blocking $\approx 22 \pm 6\%$ of the Ca^{2+} influx.

100 nM ω-conotoxin MVIIC blocked approximately 25% of the Ca^{2+} influx following depolarization with either 15 mM KCl or 1 mM 4-AP (Figure 4.5). Approximately 15-20% of the response was blocked with 10 nM toxin indicating the block was concentration-dependent.

Following stopped-flow mixing of Fura-2 loaded synaptosomes with either 15 mM KCl or 1 mM 4-AP, 10 nM ω-agatoxin IVA was effective in blocking 10% and 25% of the Ca^{2+} signal following KCl and 4-AP depolarization respectively (Figure 4.6). Increasing the toxin concentration to 100 nM did not result in a greater inhibition of the Ca^{2+} flux under either depolarization condition.

Table 4.1 presents a summary of the pharmacological characterization of the Ca^{2+} flux induced by 1 mM 4-AP or 15 mM KCl depolarization and includes data from 27.5 mM KCl conditions for comparison.

Table 4.1

4-AP or KCl Depolarization-Induced Presynaptic Ca^{2+} Influx:

Inhibition by Calcium Channel Antagonists

	Percent Inhibition	IC_{50}
1 mM 4-AP Depolarization		
nitrendipine	20%	78 nM
nimodipine	25%	63 nM
Bay K8644	N/E	
Verapamil	N/E	
ω-CONOTOXIN GVIA	22%	≈ 100 nM
ω-AGATOXIN IVA	25%	≈ 10 nM
ω-CONOTOXIN MVIIC	25%	≈ 100 nM
15 mM KCl Depolarization		
nitrendipine	N/E	
nimodipine	N/E	
Bay K8644	N/E	
Verapamil	N/E	
ω-CONOTOXIN GVIA	10%	≈ 100 nM
ω-AGATOXIN IVA	10%	≈ 10 nM
ω-CONOTOXIN MVIIC	10%	≈ 100 nM
27.5 mM KCl Depolarization		
nitrendipine	N/E	
nimodipine	15%	100 nM
Bay K8644	N/E	
Verapamil	N/E	
ω-CONOTOXIN GVIA	15%	< 100 nM
ω-AGATOXIN IVA	30%	30 nM
ω-CONOTOXIN MVIIC	40%	60 nM

N/E: no effect

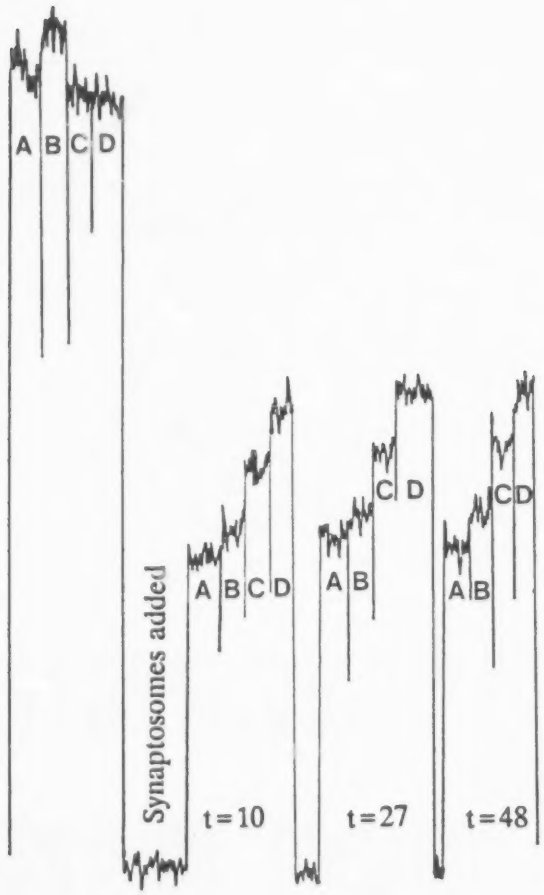


Figure 4.1 Membrane potential changes monitored with $diSC_3(5)$. 5 μL of 500 μM dye was added to 2.45 mL of nondepolarizing buffer (A), 1 mM 4-AP buffer (B), 15mM K^+ depolarizing buffer (C) or 25 mM K^+ depolarizing buffer (D). The fluorescence was monitored excitation and emission wavelengths of 622 nm and 670 nm respectively. The buffers and dye were equilibrated at 37°C and synaptosomes were added, from a thawed aliquot, to a final protein concentration of 50 μg/mL. The fluorescence change of each sample was measured at approximately 15 minute intervals for up to 2 hours.

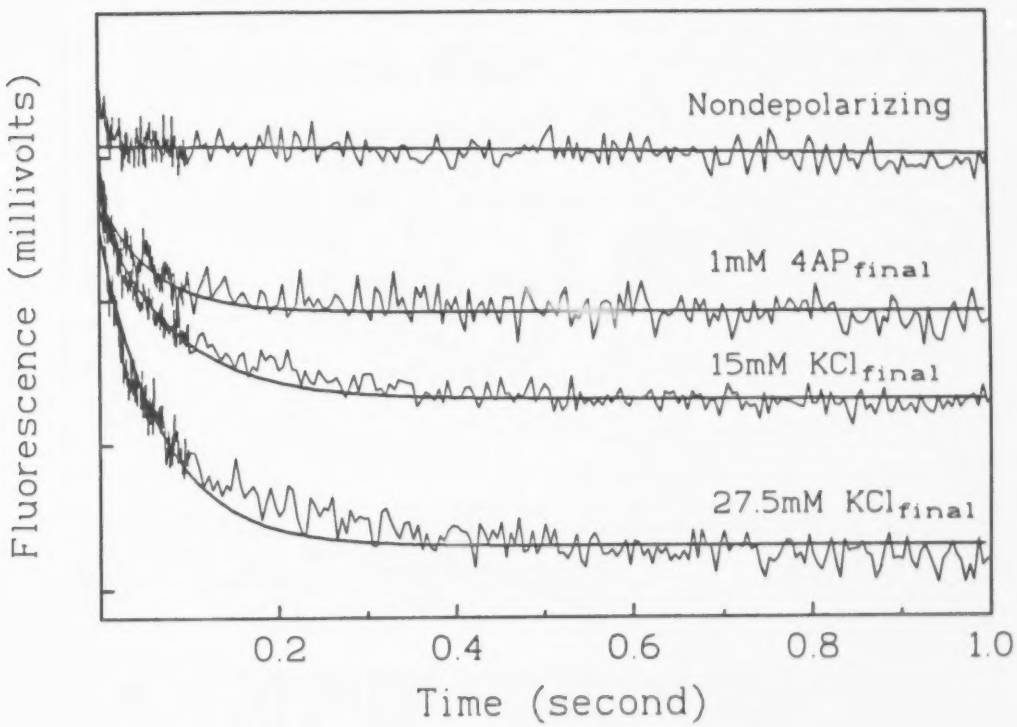


Figure 4.2 Effect of depolarization on changes in intrasynaptosomal Ca^{2+} concentration monitored by the fluorescence of Fura-2. Figure shows representative stopped-flow traces obtained upon rapid mixing of Fura-2 loaded synaptosomes with nondepolarizing (5 mM K^+) buffer and depolarizing buffer containing 25 or 50 mM K^+ or 2mM 4-AP. All buffers were osmotically balanced. The quench observed with depolarization has been fitted by a single exponential equation as described in the text.

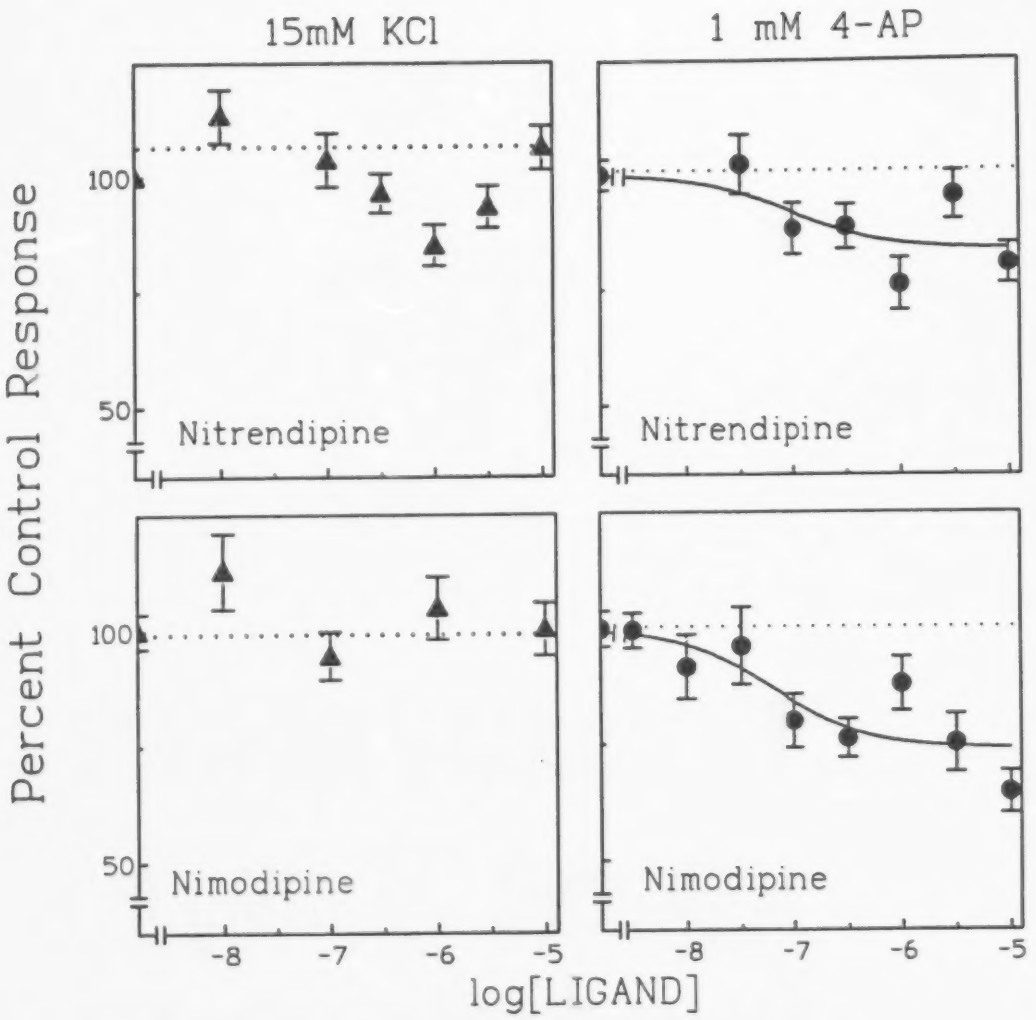


Figure 4.3 Effects of nitrendipine and nimodipine on changes in Fura-2 fluorescence following depolarization by 15 mM KCl or 1 mM 4-AP. The upper panels show nitrendipine data and the lower panels illustrate the nimodipine results. The dihydropyridine was incubated with Fura-2 loaded synaptosomes and added to mixing buffers at least 15 minutes prior to stopped-flow fluorescence measurements. The amplitude of the depolarization-induced quench at each concentration was normalized to that of control experiments in the absence of added drug. Data represent the mean and SEM of eight(KCl/nitrendipine), sixteen(4-AP/nitrendipine), four(KCl/nimodipine) and thirteen(4-AP/nimodipine) separate experiments.

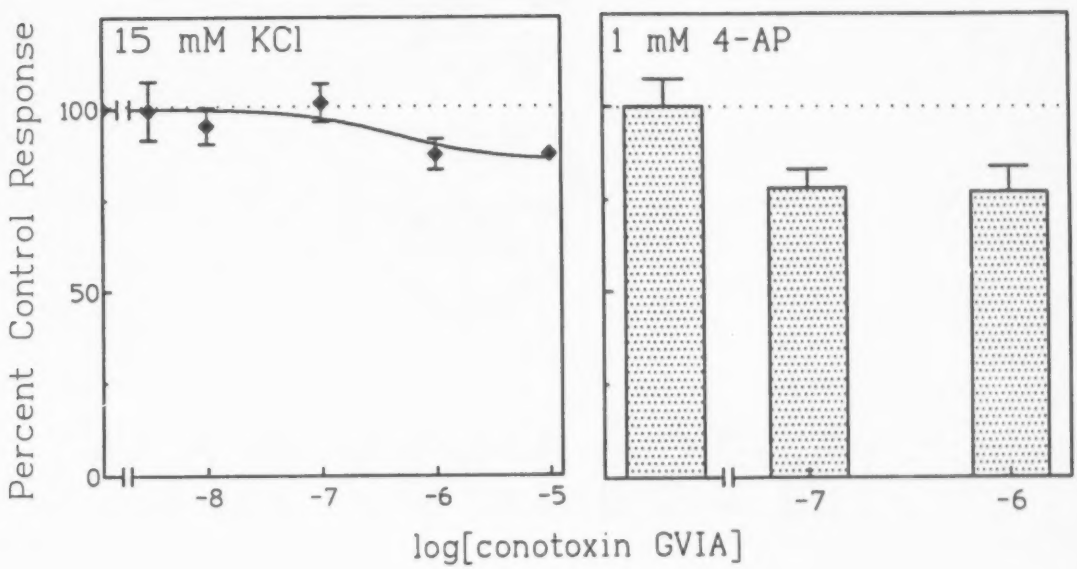


Figure 4.4 Effects of the N-type calcium channel antagonist ω-conotoxin GVIA on changes in Fura-2 fluorescence following depolarization by 15mM KCl or 1 mM 4-AP. The toxin was incubated with Fura-2 loaded synaptosomes and added to mixing buffers at least 20 minutes prior to stopped-flow fluorescence measurements. The amplitude of the depolarization-induced quench at each concentration was normalized to that of control experiments in the absence of added toxin. Data represent the mean and SEM of six separate experiments (15mM KCl) or 7-14 separate traces (4-AP).

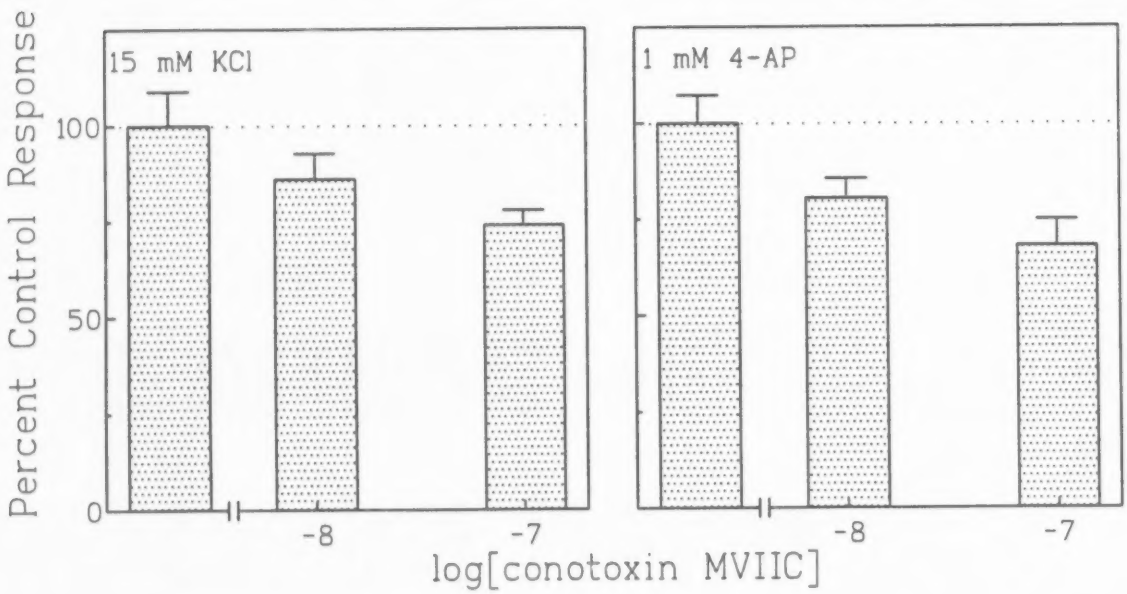


Figure 4.5 Effects of ω-conotoxin MVIIC on changes in Fura-2 fluorescence following depolarization by 15mM KCl or 1 mM 4-AP. The toxin was incubated with Fura-2 loaded synaptosomes and added to mixing buffers at least 20 minutes prior to stopped-flow fluorescence measurements. The amplitude of the depolarization-induced quench at each concentration was normalized to that of control experiments in the absence of added toxin. The data represent the mean and SEM of three separate experiments.

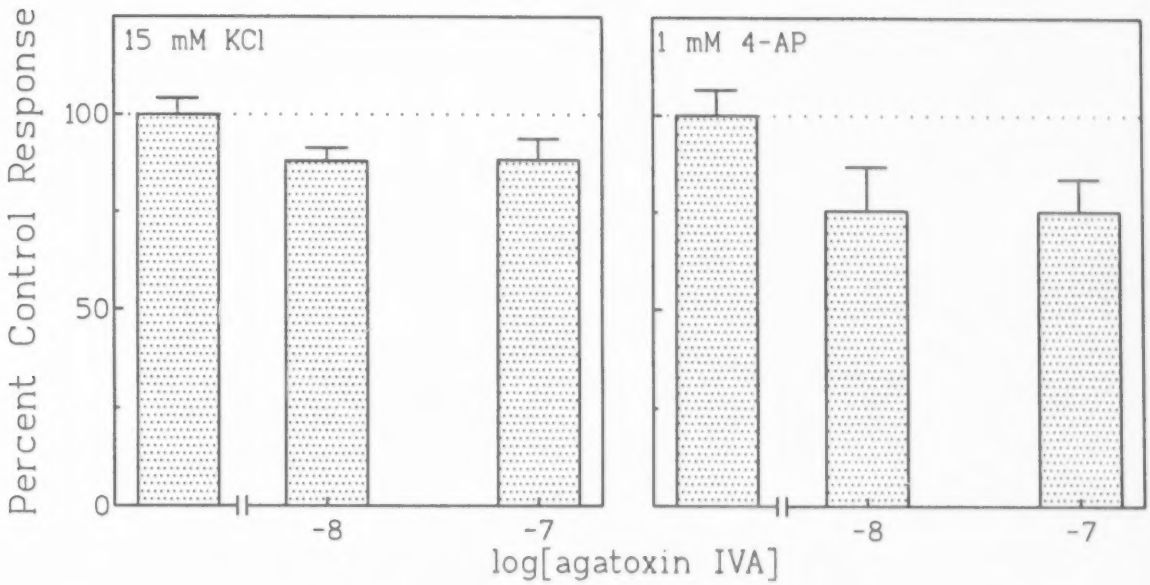


Figure 4.6 Effects of ω-agatoxin IVA on changes in Fura-2 fluorescence following depolarization by 15mM KCl or 1 mM 4-AP. The toxin was incubated with Fura-2 loaded synaptosomes and added to mixing buffers at least 20 minutes prior to stopped-flow fluorescence measurements. The amplitude of the depolarization-induced quench at each concentration was normalized to that of control experiments in the absence of added toxin. The data represent the mean and SEM of five separate experiments.

DISCUSSION

Elevating the external K^+ concentration or the addition of 1 mM 4-AP increased internal Ca^{2+} levels in synaptosomes. Although both elevated K^+ and 4-AP depolarize the synaptosomal membrane and trigger Ca^{2+} entry through voltage-dependent calcium channels, 4-AP-induced Ca^{2+} influx appears to be somewhat more sensitive to block by the dihydropyridine calcium channel antagonists, nitrendipine and nimodipine. The results observed with various animal toxins following depolarization by either an intermediate K^+ concentration or 4-AP, as compared with 27.5 mM K^+, suggest a slightly greater inhibition by ω-conotoxin GVIA, while both ω-conotoxin MVIIC and ω-agatoxin IVA blocked the signal with somewhat reduced efficacy with a lower K^+ or 4-AP depolarization protocol.

Depolarization of mammalian synaptosomes must be achieved by chemical means. The most cited method of stimulation is elevation of external $[K^+]$, which leads to a collapse of the K^+ concentration gradient and depolarization of the membrane (Nicholls, 1993; Romano-Silva *et.al.*, 1994; see Chapter 1). It has been argued that depolarization by 4-AP is a consequence of instability in the resting synaptosomal membrane potential which leads to opening of voltage-sensitive sodium channels and repetitive depolarizations (Tibbs *et.al.*, 1989a; Nicholls, 1993; see Chapter 1). Choline-substituted buffers were not used in the 4-AP experiments since activation of sodium channels is critical for depolarization and, as expected for this type of depolarization, the depolarizing effects of 4-AP were blocked in the presence of 200nM TTX, or low Na^+ buffers.

Using the membrane potential dye $diSC_3(5)$, it was not possible to detect changes in the membrane potential between nondepolarized and 1 mM 4-AP stimulated synaptosomes (Figure 4.1). Others however, using radiolabelled cation indicators, have reported a redistribution of phosphonium ions or $^{86}Rb^+$ following 4-AP depolarization of synaptosomes that was equivalent to a depolarization of 5-15 mV (Tibbs *et.al.*,1989a).

Several subtypes of calcium channels have been pharmacologically identified with the presynaptic terminal (Dunn, 1988; Hillyard *et.al.*, 1992; Mintz *et.al.*, 1992; Turner *et.al.*, 1992; 1993; Uchitel *et.al.*, 1992; Yasuda *et.al.*, 1993; Wheeler *et.al.*, 1994;

Maubecin *et.al.*, 1995). Although non-L and non-N subtypes appear to be most functionally significant in studies of Ca^{2+} influx and neurotransmitter release (Turner *et.al.*, 1993; Wheeler *et.al.*, 1994; Maubecin *et.al.*, 1995), high affinity binding sites for antagonists of L and N-type channels are present in synaptosomes (Dunn, 1988; Yasuda *et.al.*, 1994). It may be that these proteins do not function as voltage-dependent calcium channels, or it may be possible that certain stimulating conditions such as the degree or frequency of depolarization or other external conditions are necessary to activate these channels (Middlesmiss & Spedding, 1985; Dunn, 1988).

Depolarization induced by elevating the external K^+ concentration to 15 mM gave rise to Ca^{2+} influx generally unaffected by the dihydropyridine antagonists nitrendipine and nimodipine. In contrast, 4-AP-induced Ca^{2+} influx was blocked by approximately 25% by each drug (Figure 4.3). The differences in the depolarization induced (sustained versus repetitive), by the two methods of stimulation may account for the differences in dihydropyridine sensitivity.

In cardiac and smooth muscle tissue, 1,4-dihydropyridine drugs demonstrate voltage-dependent activity while verapamil has a frequency dependent mode of activity on the L-type channel (Triggle, 1994; Uski *et.al.*, 1994). The voltage dependence of 1,4-dihydropyridines is demonstrated by a higher affinity of binding to depolarized cardiac and smooth muscle tissues (reviewed by Janis & Triggle, 1991). The results reported here, in neuronal tissue, suggest a possible frequency dependence in activity of nitrendipine and nimodipine, as a greater sensitivity to these dihydropyridines is apparent following depolarization by 4-AP. However, the difference may also reflect the more moderate depolarization induced by the latter method. In a previous report, the effect of depolarization on dihydropyridine binding and dihydropyridine modification of $^{45}Ca^{2+}$ influx in synaptosomes was evaluated (Dunn, 1988). Binding and modulation of influx was found to be voltage-sensitive, both being decreased at higher levels of depolarization. Interestingly, the voltage-dependence observed with these synaptosome studies indicate a greater sensitivity to 1,4-dihydropyridines at lower levels of depolarization, in contrast to observations of dihydropyridine voltage-dependence made in other tissues (Janis & Triggle, 1991).

Bay K8644, an L-type calcium channel agonist was without effect in altering the Fura-2 fluorescence in synaptosomes. Others have reported increased $^{45}Ca^{2+}$ influx (White & Bradford, 1986; Dunn, 1988) in the presence of this drug. The discrepancies may arise from the use of racemic mixtures of Bay K8644. In cardiac and smooth muscle tissue, the S-enantiomer of this drug has been shown to exert agonist activity while the R-enantiomer acts antagonistically (Triggle & Janis, 1987). Thus the racemic mixture used in the present study may have masked any specific effects.

Verapamil, a phenylalkylamine L-type calcium channel antagonist, was also ineffective in blocking Ca^{2+} influx in cortical synaptosomes depolarized by either elevated K^+ or 4-AP. Verapamil has a clear frequency-dependent mode of activity on L-type calcium channels in the heart, making it a particularly effective drug for treatment of cardiac arrhythymias (Janis & Triggle, 1991; Uski *et.al.*, 1994). However other synaptosome studies with verapamil indicate very high concentrations of the drug are necessary in order to observe an effect on either $^{45}Ca^{2+}$ influx or transmitter release (Dunn, 1988; Adamson *et.al.*, 1989; Canzoniero *et.al.*, 1993) and at these concentrations effects on Ca^{2+} influx have been shown to be due to verapamil effects on the Na^+-Ca^{2+} exchanger (Canzoniero *et.al.*, 1993).

The antagonistic effects of ω-conotoxin GVIA, ω-conotoxin MVIIC and ω-agatoxin IVA on presynaptic Ca^{2+} flux differed slightly under the depolarization stimuli tested. ω-Conotoxin GVIA was slightly more effective in inhibiting Ca^{2+} influx, suggesting that 4-AP depolarization may stimulate more Ca^{2+} influx through N-type calcium channels in rat brain synaptosomes. Submicromolar concentrations of ω-conotoxin MVIIC blocked 25% of the presynaptic Ca^{2+} flux measured in the current studies as compared to 40% inhibition following 27.5 mM KCl depolarization. ω-Agatoxin IVA, did not demonstrate marked differences in sensitivity of block following different stimulation protocols, although the extent of block did appear somewhat reduced following 15 mM KCl as compared with 1 mM 4-AP or 27.5 mM KCl depolarization. Different subtypes of calcium channels as measured electrophysiologically in tissues (Miller & Fox, 1990), or in artificial bilayers with purified and reconstituted channels (De Waard *et.al.*, 1994), or in cultured cells specifically expressing different α_1 calcium

channel subunits (Zhang *et.al.*, 1993), exhibit distinct voltage dependent activation characteristics. With different depolarization protocols slight differences in the contribution of different subpopulations of calcium channels to the Ca^{2+} influx at the terminal may be expected.

There is evidence that the depolarization induced in presynaptic terminals by elevated $[K^+]$ or 4-AP results in distinct patterns of Ca^{2+} influx (Tibbs *et.al.*, 1989b; Nicholls, 1993) and different degrees of endogenous or radiolabelled neurotransmitter release (Nichols *et.al.*, 1994). In the current study nitrendipine was shown to block 20-25% of the Ca^{2+} influx in 4-AP depolarized synaptosomes while demonstrating no block in K^+ stimulated conditions.

It is not confirmed that 4-AP depolarization of synaptosomes is "more physiological", however differential sensitivity to certain calcium channel antagonists following distinct methods of chemical induced depolarization is suggested by these results. Continued study is likely to clarify the basis of the differential responses of the presynaptic terminal to these stimulation protocols.

Chapter Five

Effects on Presynaptic Calcium Channels of Immunoglobulins Purified from Amyotrophic Lateral Sclerosis (ALS) Patients

RESULTS

In order to determine if IgG purified from ALS patients has any specific effect on presynaptic calcium channels, the synaptosomal model system was used in a series of experiments to investigate the effects of IgG from eight patients with sporadic ALS. This work was done in collaboration with Dr. Michael Brooke in the Department of Neurology, University of Alberta.

Table 5.1 summarizes the available clinical history of the eight ALS patients from whom IgG was purified. The average age of the ALS patients was 55.4 $\pm$ 4.5 years. The non-ALS, non-neurological disease control IgG was purified from blood collected from relatively young, healthy adults (average age = 27.0 $\pm$ 2.1 years).

The individual subject IgG samples were tested at 3 or 4 protein concentrations between 1 μg/mL and 1 mg/mL. Due to the limited supply of IgG, the highest concentration was not tested with all subject ALS samples. Figure 5.1 shows the results for three concentrations of IgG with a depolarization stimulus of 15 mM KCl. In order to evaluate the possible effects of a stronger depolarizing stimulus on the interaction of ALS IgG and presynaptic Ca^{2+} flux, the eight ALS samples were tested in the presence of a final potassium concentration of 27.5 mM. The results are presented in Figure 5.2.

Although some ALS samples consistently resulted in an increased amplitude (i.e. "2" in the 15 mM KCl stimulation and "5" and "8" in the 27.5 mM KCl stimulation) the effect does not appear to be dependent on the amount of IgG present, nor consistent under different depolarization conditions. Rigorous statistical analysis of the data was not completed because the results between most ALS samples were quite variable and no logical rationale based on the patient history (Table 5.1) permitted select grouping of the ALS samples. The results for the non-ALS IgG samples under both conditions of

stimulation are also presented in Figures 5.1 and 5.2. Control experiments using IgG purified from ALS subject "5" and one of the non-ALS subjects were carried out in which the IgG was inactivated by heating to 95°C for 20 minutes. No effect was observed on either the membrane potential response or the stopped-flow fluorescence signal.

Table 5.1

Clinical Data of ALS Patients

ALS #	Sex	Age	M	other
1	M	45	>14	no other known medical problems
2	F	44	2	family history of diabetes and thyroid disease
3	F	38	7	difficult to definitively diagnose; accident trauma when young
4	M	53	4	sibling died of ALS; slow progression
5	F	59	2	bulbar onset; moderate progression
6	F	75	11	no bulbar symptoms; slow progression
7	M	60	13	difficult to definitively diagnose
8	M	69	9	bulbar onset; rapid progression
		55.4 ±4.5	7.8± 1.7	

* "M" indicates "months diagnosed" prior to collection of blood sample; Age is in years.

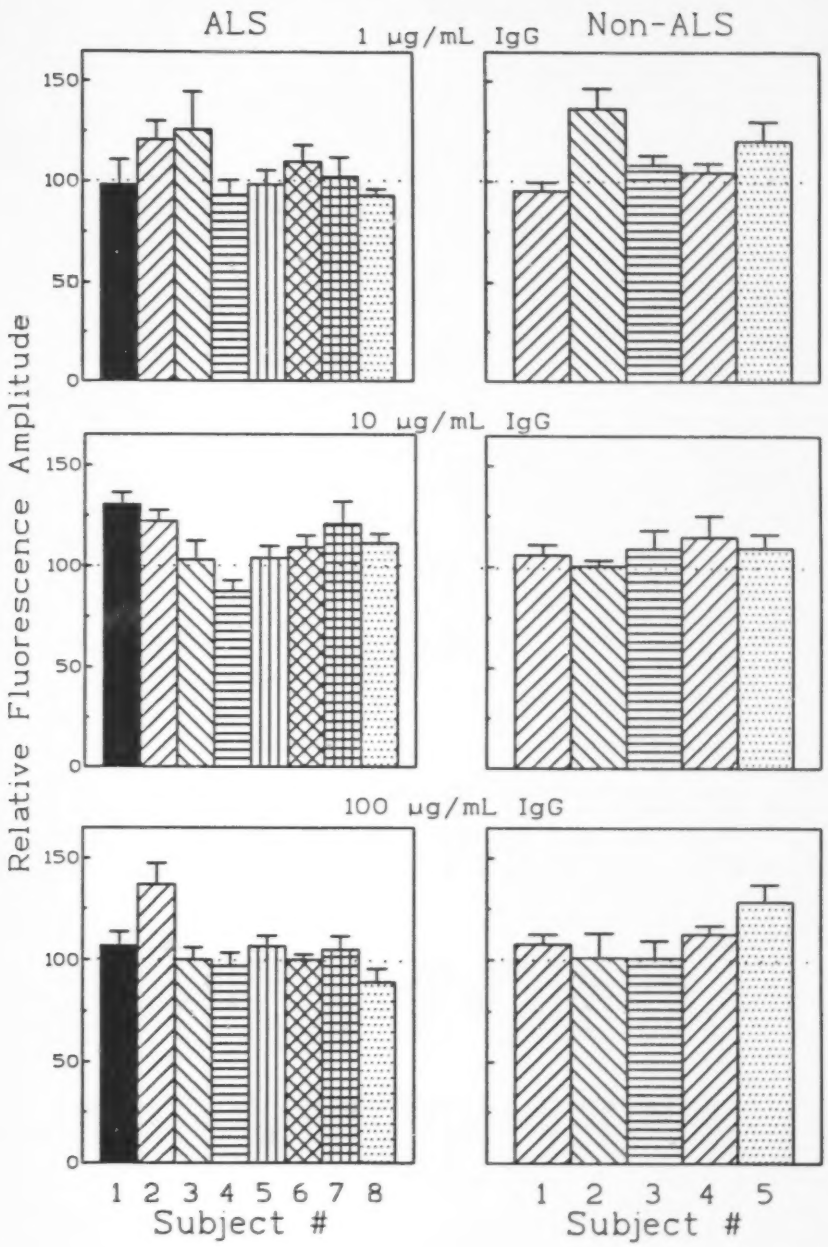


Figure 5.1 Effect of ALS patient and control subject IgG on presynaptic Ca^{2+} influx induced by 15 mM KCl depolarization. The data are presented as the change in the stopped-flow signal amplitude relative to the amplitude measured in the absence of any immunoglobulins. 1, 10 and 100 µg/mL IgG protein concentrations were tested as indicated. The individual results shown are the mean and SEM of data from 2-5 separate stopped-flow experiments.

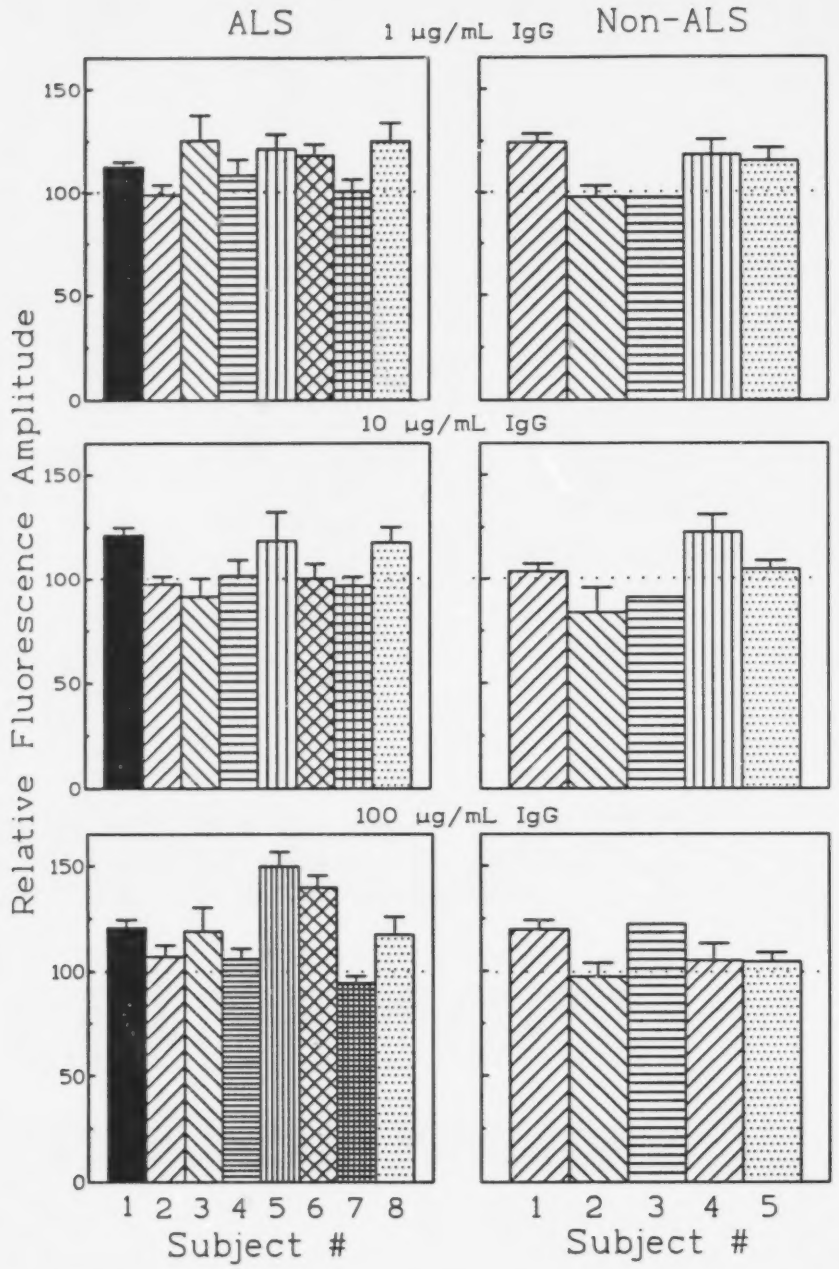


Figure 5.2 Effect of ALS patient and control subject IgG on presynaptic Ca^{2+} influx induced by 27.5 mM KCl depolarization. The data are presented as the change in the stopped-flow signal amplitude relative to the amplitude measured in the absence of any immunoglobulin. 1, 10 and 100 μg/mL IgG protein concentrations were tested as indicated. The individual results shown are the mean and SEM of data from 2-5 separate stopped-flow experiments.

DISCUSSION

ALS is a progressive, degenerative human disease in which upper and lower motor neuron function is lost, leading to muscle weakness, paralysis and eventual death due to respiratory failure (Appel & Stefani, 1991). In approximately 10% of ALS patients, the incidence is familial and in some cases progress has been made in identifying the defective gene (Rosen *et.al.*, 1993). The majority of individuals suffering from ALS have a "sporadic" form of the disease and several hypotheses of the pathogenesis of this form have been proposed, including autoimmunity (Appel & Stefani, 1991; Rowland, 1991).

In the current study, IgG purified from eight ALS-diagnosed individuals did not significantly alter the presynaptic Ca^{2+} signal measured using stopped-flow fluorimetry. Three IgG protein concentrations and two levels of depolarization were tested and no systematic change was observed with any ALS IgG sample. Although these findings are not directly reconcilable to voltage-dependent calcium channel autoimmunity being the primary factor in the etiology of ALS, the lack of clear effect in our findings does not negate the possibility of an immunological role in this disease.

ALS is recognized as a complex neurodegenerative disease and a definitive diagnosis requires excluding several other possible disease processes. There is a diversity in the age of onset, symptomology, and rate of progression of ALS. Recent reviews have evaluated several possible causal factors (Rowland 1991; Appel *et.al.*, 1993; Smith & Appel, 1995; Eisen, 1995; Antel & Cashman, 1995). There is some speculation that ALS is not in fact a single disease process but the outcome of motorneuron degeneration brought on by a variety of mechanisms (Eisen, 1995).

Initial support for an autoimmune mechanism in the progression of ALS was provided by the finding that a syndrome similar to human ALS can be experimentally induced by transfer of serum or purified IgG. Guinea pigs injected with tissue extracts from cortical gray matter of a second species develop immunoglobulins. These antibodies were purified and injected into mice, and it was observed that miniature endplate potential frequency increased at the neuromuscular junction. The changes were similar to changes observed following injection of immunoglobulins purified from ALS

patients (Engelhardt *et.al.*, 1990; Appel *et.al.*, 1991). Subsequent work using various experimental approaches from this group provided considerable support for presynaptic calcium channels being the target antigen of the ALS IgG (reviewed in Appel *et.al.*, 1993; Llinás *et.al*, 1993; Smith *et.al.*, 1994; Kimura *et.al.*, 1994). Additional work by Nistri and co-workers (Zhainazarov *et.al.*, 1994) reported that IgG or serum fractions from ALS-diagnosed individuals reduced the Ca^{2+} current measured in cultured granule cells. Of particular interest was the demonstration that ALS IgG acted directly on P-type calcium channels (Llinás *et.al.*, 1993). In this study P-type channels were isolated from guinea pig cerebellum on an sFTX affinity column and reconstituted in a lipid bilayer. Single channel recordings indicated that ALS IgG increased the channel conductance; while patch-clamp recordings from Purkinje cells showed that the increased Ca^{2+} current could be blocked by ω-agatoxin IVA and FTX. P-type calcium channels are involved in mediating presynaptic Ca^{2+} flux in our preparation (Chapter Three), thus this was used as a model to further investigate the role of immunoglobulins from ALS patients on calcium channel function.

The results of the present study do not demonstrate either an enhancement or attenuation of presynaptic Ca^{2+} flux in the presence of IgG purified from eight ALS-diagnosed individuals. There are a number of possible explanations.

The IgG purified from these eight individuals may not contain antibodies directed against voltage dependent calcium channels. A specific assay to confirm the presence of such antibodies was not completed due to the lack of functional effects observed in the stopped-flow studies. It is also possible that calcium channel specific antibodies are present in the ALS IgG but the interaction of the antibody does not lead to a change in the channel function.

Unlike the majority of previous reports on the interaction of ALS IgG on voltage-sensitive calcium channels, in the current report, the purified IgG fractions were not pooled together. It is possible that all the IgG samples contributing to the pooled supply do not individually demonstrate the effect observed. In reports where individual ALS IgG samples were clearly used separately, 75% of the ALS IgG samples tested

positively for immunoreactivity with the L-type calcium channel purified from rabbit skeletal muscle in an enzyme-linked immunoassay protocol (Smith *et.al.*, 1992). Specific labelling of the α_1 subunit of skeletal muscle L-type channels by purified IgG was reported for 8 of 12 ALS subjects (Kimura *et.al.*, 1994). Likewise, a decrease in the cultured granule cell Ca^{2+} current was observed with 3 of 4 patient immunoglobulin samples (Zhainazarov *et.al.*, 1994). The one ALS sample that was without effect in this study was from an individual on immunosuppressant therapy, while both IgG and IgM fractions of another individual significantly decreased the current. Thus a uniform effect of all ALS IgG samples on voltage-dependent calcium channels has not been observed in previous work. Indeed it has been suggested that autoimmunity to calcium channels in ALS may be one of several factors contributing to a very diverse disease process and therefore may not be a significant element in every case (Eisen, 1995). The current results may reflect this diversity and indicate that IgG directed against voltage-dependent calcium channels is not a significant factor for the eight ALS individuals examined.

It is possible that this synaptosome model system is not sufficiently sensitive to detect the changes in calcium channel function effected by ALS IgG. In the pathophysiology of ALS, upper and lower motoneurons are primarily affected and it is possible that significant differences exist between central nervous system nerve terminals and the primary site of ALS nerve degeneration in the periphery. Although P-type channels may contribute in an autoimmune mechanism of ALS (Llinás *et.al.*, 1993; Smith *et.al.*, 1994), possible L-type channel involvement has also been indicated (Delbono *et.al.*, 1991; Smith *et.al.*,1992 Magnelli *et.al.*, 1993; Kimura *et.al.*, 1994). L-type calcium channels have not been shown to be abundantly present on CNS presynaptic nerve endings, thus the proportion of target antigen present in the synaptosome preparation may not be sufficient to provide a satisfactory signal to background Ca^{2+} flux measurement. It is also possible that the IgG purified from ALS patients is directed against calcium channels, but differences between human and rat calcium channel proteins reduces the liklihood of the antibody-antigen interaction. Cloned calcium channel α_1 subunits from rat, rabbit and human tissues are reported to be 40-70%

homologous in their cDNA primary sequences (reveiwed by Hofmann *et.al.*, 1994). It is not yet possible to determine the degree of homology between entire calcium channel proteins from different tissues. Although species differences in protein structure do exist, others have reported that human ALS IgG does affect calcium channel function in the rat (Smith *et.al.*, 1994; Zhainazarov *et.al.*, 1994), mouse (Appel *et.al.*, 1991) and guinea pig (Llinás *et.al.*, 1993).

The link between antibodies directed against voltage-dependent calcium channels and the pathological course of ALS is unclear. The primary site of neural degeneration in this disease are the upper and lower motor neurons (Appel & Stefani, 1991; Rowland 1991) and the mechanism by which circulating antibodies would be specifically directed against calcium channels at these synapses is not readily apparent. Accounting for the particular localization of the disease process in ALS has been a persistent difficulty with any of the proposed etiologies of this disease (Smith & Appel, 1995).

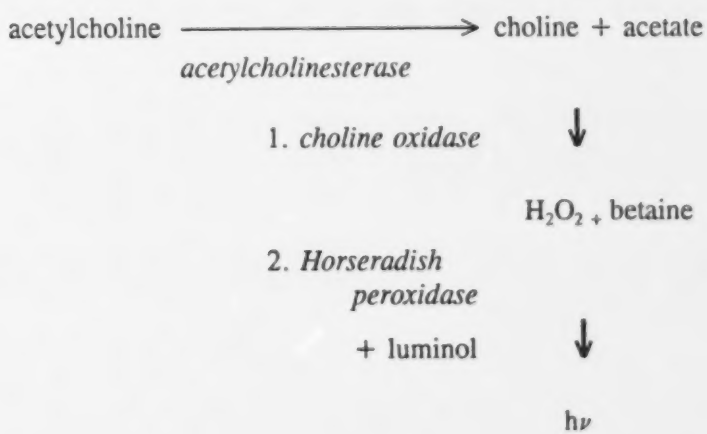
The current results do not strongly support a definitive role for calcium channel-directed IgG in the pathogensis of ALS. The IgG purified from the eight ALS patients had no systematic effect on Ca^{2+} influx through voltage-sensitive, presynaptic calcium channels. These results most likely reflect the fact that many, and possibly different factors for any individual, combine in the development of ALS.

Chapter Six
Neurotransmitter Release Studies

In order to develop a rapid assay for measuring neurotransmitter release from synaptosomes, several different approaches were investigated. The methodology and results obtained with (a) three fluorescent assays;(b) a rapid sampling method with high performance liquid chromatography analysis of neurotransmitters, and (c) dual-labelling of the synaptosome transmitter stores with [^{3}H]GABA and [^{14}C]glutamate, are presented.

Fluorescence Protocols - Acetylcholine, glutamate and GABA: Three fluorescent assays were evaluated as possible methods of measuring endogenous neurotransmitter release.

1. Acetylcholine (ACh) Luminescent Assay - Israel and Lesbats (1981) have developed a chemiluminescent method for measuring ACh release using luminol. The reactions proceed as shown below.



In a solution containing acetylcholinesterase, any ACh present will be converted to choline and acetate. The assay is based on the conversion of choline to betaine and H_2O_2 catalyzed by choline oxidase. In the presence of peroxidase and luminol, a chemiluminescent reaction between the luminol and the H_2O_2 will produce a light

response proportional to the amount of choline originally present. Since the choline originates from the ACh, the luminescent response is also proportional to the original ACh present.

Preliminary studies using the Applied Photophysics Ltd. SF.17MV Microvolume Stopped-Flow Fluorimeter were carried out. Stock solutions of 100 units/mL choline oxidase, 2 mg/mL horseradish peroxidase type II and 1000 units/mL acetylcholinesterase (all from Sigma) were prepared in distilled H_2O. A 1 mM stock solution of luminol was made by dissolving the powder in a few drops of 1 M NaOH and diluting with 0.2 M Tris buffer (pH=8.6). All stock solutions were divided into appropriate volumes as necessary for subsequent experiments and stored at -86°C until use.

Stopped-flow experiments were carried out at room temperature. Immediately prior to an experiment, an "assay mixture" was prepared from the stock solutions of the reagents. The assay mixture contained 100 μL choline oxidase, 50 μL horseradish peroxidase, 100 μL luminol in a total volume of 2.5 mLs of phosphate buffer (pH 8.6). For calibration studies, an aliquot of assay mixture was rapidly mixed with a equal volume of H_2O_2. In some experiments ACh was added to the assay mixture and mixed with an equal volume of acetylcholinesterase ($\approx$16 units/mL) that was prepared in phosphate buffer. After mixing all luminescence was recorded through a twelve stage end window photomultiplier and analyzed using an Archimedes computer and the Applied Photophysics Ltd. kinetic software package.

RESULTS AND DISCUSSION

Figure 6.1 illustrates the stopped-flow traces obtained after the assay mixture was mixed with different solutions of H_2O_2. A clear luminescent signal was measurable for H_2O_2 concentrations of 1 μM or more but submicromolar concentrations were not detectable. Decreasing the delay between the preparation of the assay mixture and recording of the stopped-flow signals did not change the sensitivity. In addition, no measurable changes in luminescence were observed when 50 pM or 1 μM ACh were added to the assay mixture and this solution was mixed with a second buffer containing acetylcholinesterase.

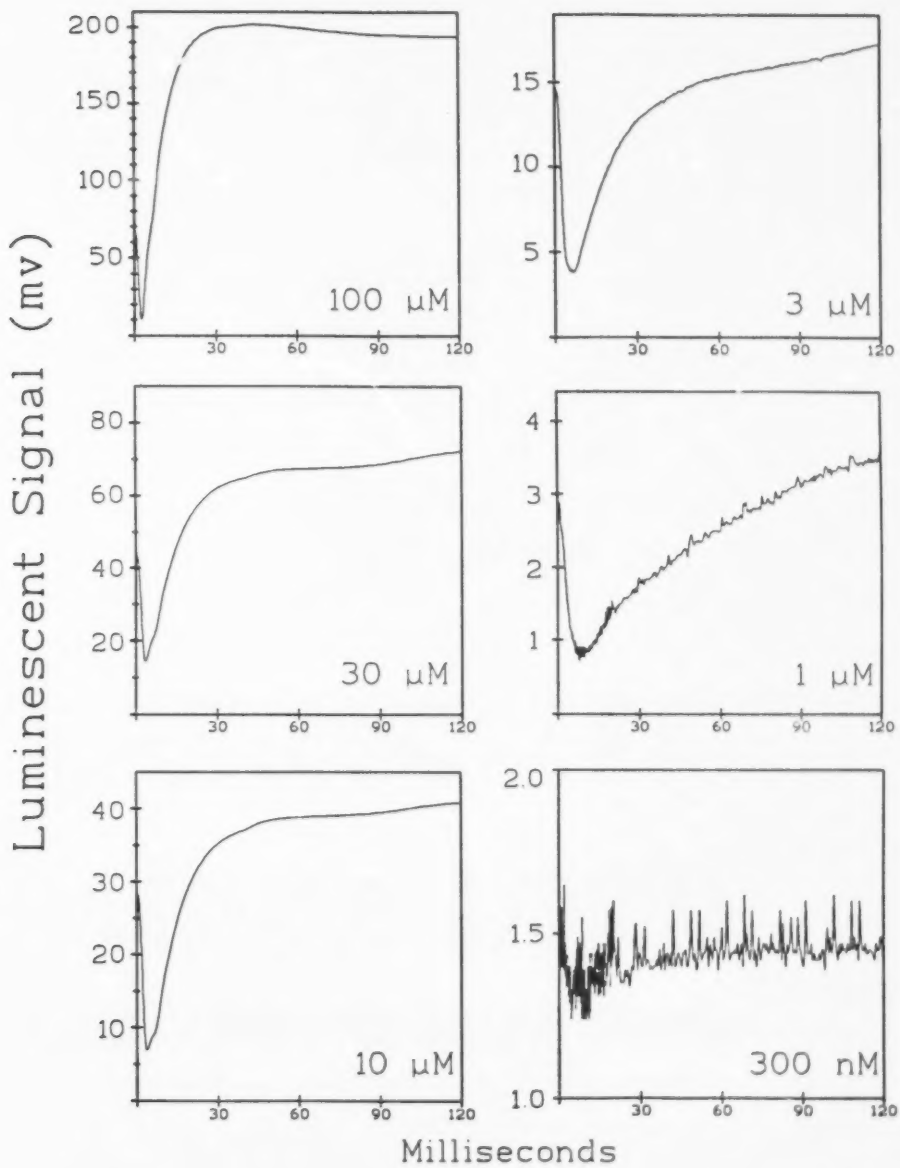


Figure 6.1 Stopped-flow traces from luminol/H_2O_2 assays. The "assay mixture" and the indicated concentrations of H_2O_2 were mixed in the stopped-flow fluorimeter. Note the different ordinate scales for the different conditions.

In the reaction of choline with choline oxidase, one molecule of H_2O_2 is produced for each molecule of choline oxidized and thus the concentration of H_2O_2 detectable indicates the sensitivity of the assay for measurement of ACh. Unfortunately sensitivity of only micromolar concentrations as obtained in these test studies, would not be adequate for measuring ACh released from cortical synaptosomes. Measurement release from synaptosomes is further complicated by the necessity to control for non-ACh linked choline release, which can be significant (C. Benishin, personal communication). Cholinergic synapses represent only 10-15% of cortical synapses and literature reports of the level of ACh released from mammalian tissue indicate that a technique to monitor this transmitter would require picomolar sensitivity (Říčný *et.al.*, 1989).

The iimited sensitivity observed in these studies is most likely primarily due to the limitations of the photomultiplier tube within the Applied Photophysics SF-17MV system. Other groups that have used this luminescence assay to measure ACh have utilized a luminometer, which is an instrument designed to detect very low levels of light production. The preferred type of photomultiplier tube has a rubidium-bialkali cathode which provides a quantum efficiency of up to 28% in the visual range. More common photomultiplier tubes have bialkali cathodes and a much lower quantum efficency ($\approx$3%). In addition, the photomultiplier should be operated in the photon counting mode as opposed to the direct current mode in order to increase the signal to noise ratio (Berthold & Maly, 1987). Additional electronic filtering of the signal may also improve the usefulness of this technique (P. Thomas, personal communication; Thomas, 1987).

An additional modification that would be necessary if one were to use this protocol would be to adjust the conditions so that the pH of the basic assay (pH = 8.5) could be reduced for use with intact synaptosomes. Other workers have been able to modify the method to record the luminescence at a pH of 7.7 to 8.0 but report a decrease in the luminescent signal (Diebler, 1982). Lundy and co-workers have used a modification of this assay in studies of ACh release from chicken and rat brain slices (Lundy *et.al.*, 1989; Lundy *et.al.*, 1991); however the difficulties in obtaining reproducible results prompted this group to forego further investigations using this

technique (P. Lundy, personal communication). Due to these inherent difficulties with this assay for ACh, methods for monitoring the release of other neurotransmitters were investigated.

2. *Fluorescent Glutamate Dehydrogenase Assay* - The conversion of glutamate to 2-oxoglutarate by glutamate dehydrogenase results in the reduction of nicotine adenine dinucleotide phosphate ($NADP^+$) to NADPH.

$$\text{glutamate} + NADP^+ \xrightarrow[\textit{glutamate dehydrogenase}]{} \text{2-oxyglutarate} + NADPH + H^+$$

The production of NADPH can be monitored fluorimetrically (Lowry *et.al.*,1957). Nicholls and co-workers have developed this technique to measure endogenous glutamate release from cerebrocortical synaptosomes (Nicholls & Sihra, 1986; Nicholls *et.al.*, 1987).

Preliminary studies using a Perkin-Elmer MPF4 fluorimeter were carried out to establish suitable assay conditions. Stock solutions of 20 mM $NADP^+$ (Sigma), ≈55 units/mL L-glutamate dehydrogenase (Sigma) and glutamate were prepared in nondepolarizing buffer. The enzyme was dialysed against dH_2O to remove contaminating glutamate prior to preparation of the stock solution (Nicholls *et.al.*, 1987). Frozen synaptosomes were thawed and diluted to 0.67 mg/mL in nondepolarizing buffer. The diluted synaptosomes were placed in quartz fluorimeter cuvettes and allowed to equilibrate to 37°C with stirring. $NADP^+$ was added to a concentration of 100 μM. Two minutes after the $NADP^+$ addition, ≈15 units L-glutamate dehydrogenase was added and fluorescence was recorded with excitation and emission wavelengths of 330 nm and 460 nm respectively. The initial fluorescence signal is due to the reduction of external and residual glutamate. Following the plateau of this initial signal, the synaptosomes were depolarized by presentation of 30 mM KCl. Fluorescence was recorded and, at the end of each experiment, addition of known glutamate concentrations allowed calibration of the fluorescence signal.

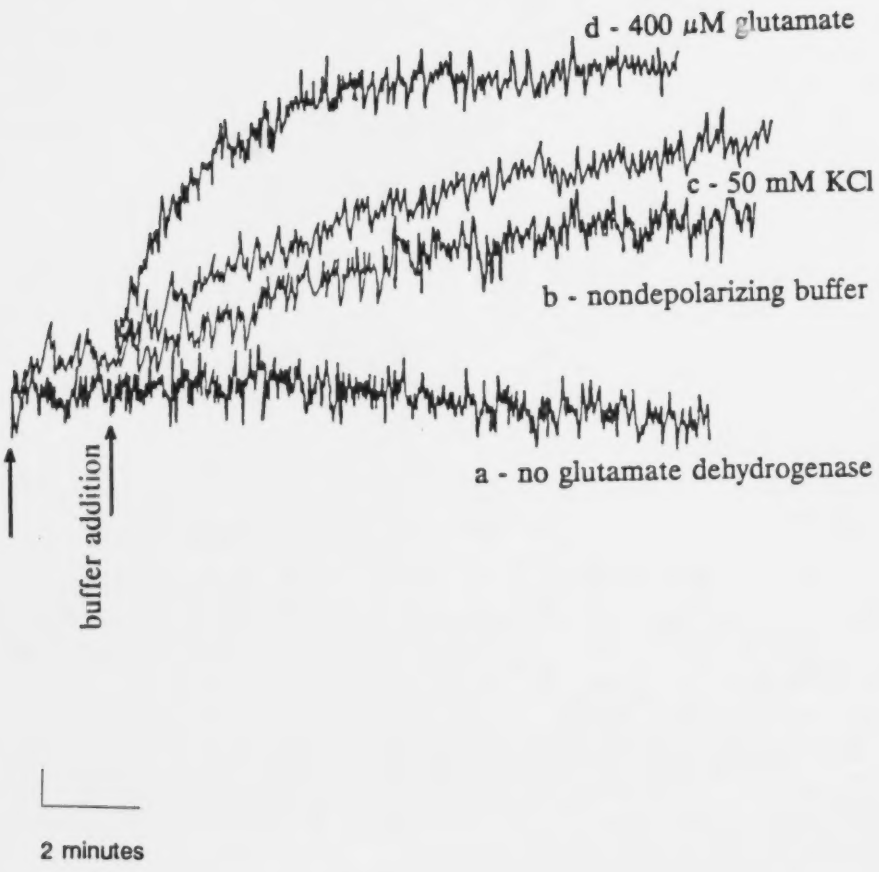


Figure 6.2 Fluorescence traces from one glutamate dehydrogenase assay. At the first arrow glutamate dehydrogenase was added to all synaptosome solutions, except (a), as outlined in the text. After a two minute incubation, 75μL of nondepolarizing buffer was added to samples (a) and (b); 75μL of 1M KCl (final $[K^+]$ = 50mM) was added to sample (c); and 75μL of 400 μM L-glutamate was added to sample (d).

RESULTS AND DISCUSSION

The results of a fluorimetric assay with synaptosomes are shown in Figure 6.2. In the absence of $NADP^+$, no enhancement of the fluorescence signal was observed (trace a). When the dinucleotide was present, an increase in fluorescence was observed due to the conversion of extrasynaptosomal glutamate to 2-oxoglutarate (trace b). On addition of KCl to increase the K^+ concentration to 50 mM KCl, the fluorescence enhancement was measurably greater than that observed in the absence of added K^+ (trace c).

Fluorimetric detection of glutamate released from mammalian central nervous system preparations is a well established technique that is used by several groups (McMahon & Nicholls, 1991; Herrero *et.al.*, 1992; Romano-Silva *et.al.*, 1994; Nichols *et.al.*,1994; Terrian, 1995). The preliminary fluorescence studies carried out suggest that this method has the necessary sensitivity and, as discussed elsewhere (Nicholls, 1989), the technique has several advantages. However, the time resolution of this assay is only tens of seconds to minutes, being limited by the rate at which glutamate dehydrogenase converts glutamate to 2-oxoglutarate (Nicholls & Sihra, 1986), a process far slower than the rate at which transmitter is actually released. In view of the more rapid method of measuring changes in intrasynaptosomal $[Ca^{2+}]$ that had been developed (Chapter Three), it was desirable to develop a technique for measuring neurotransmitter release that would have a time-resolution that more closely approached the sub-second resolution achievable with stopped-flow fluorimetry.

3. Fluorescent GABase Assay - The amount of GABA present in a solution containing GABA-transaminase and succinic semialdehyde dehydrogenase can also be monitored fluorimetrically by measuring the reduction of $NADP^+$ to NADPH (Hirsch & Robins, 1962). The chemical basis of the reaction is the conversion of GABA to succinic semialdehyde by GABA-transaminase and the subsequent oxidation of succinic semialdehyde to succinate by succinic semialdehyde dehydrogenase. In the latter step $NADP^+$ is reduced to NADPH.

GABA + α-ketoglutarate $\xrightarrow[\textit{GABA-transaminase}]{}$ **succinic semialdehyde + glutamate**

succinic semialdehyde + $NADP^+$ + H_2O $\xrightarrow[\textit{succinic semialdehyde dehydrogenase}]{}$ **succinate + NADPH + H^+**

The protocol described below utilized gabase, an enzyme solution containing both GABA-transaminase and succinic semialdehyde dehydrogenase.

Unlike the fluorimetric assay for monitoring glutamate, this protocol cannot assay GABA in a continuous manner because discrete samples must be collected and processed through a number of steps before the fluorescence signal due to the presence of GABA can be quantitated.

A 5 unit/mL stock solution of gabase (Sigma) was prepared in 0.075 M potassium phosphate, 25% (v/v) glycerol buffer, pH=7.2. Aliquots were stored at -86°C until use. Solutions of 0.7% β-mercaptoethanol, 10 mM $NADP^+$ and 50 mM α-ketoglutarate were prepared in Boehringer buffer (1 M Tris/HCl; pH 7.9). Phosphate (0.5 M Na_3PO_4/0.7 M K_2HPO_4) and 10 M NaOH/0.15% H_2O_2 buffers were also prepared. The assay "mix" was made fresh prior to each experiment and kept on ice throughout. The mix contained 50 μL Boehringer buffer, 5 μL β-mercaptoethanol, 5 μL NADP+, 10 μL α-ketoglutarate, 25 μL gabase and 10 μL dH_2O.

Experimental samples were prepared by adding 5 μL "mix" to 25 μL of dH_2O (blank conditions), GABA standards or synaptosome release samples. These solutions were shielded from light and allowed to stand at room temperature for 45 minutes. After this incubation, 25 μL of phosphate buffer was added and the mixtures were vortexed and heated at 60°C for 15 minutes. 100 μL of the NaOH/H_2O_2 solution was added to each sample and heating was continued for a further 10 minutes. The solutions were transferred to quartz fluorimeter cuvettes and 800 μL dH_2O was added. Fluorescence was recorded using a Perkin-Elmer MPF4 fluorimeter with excitation and emission

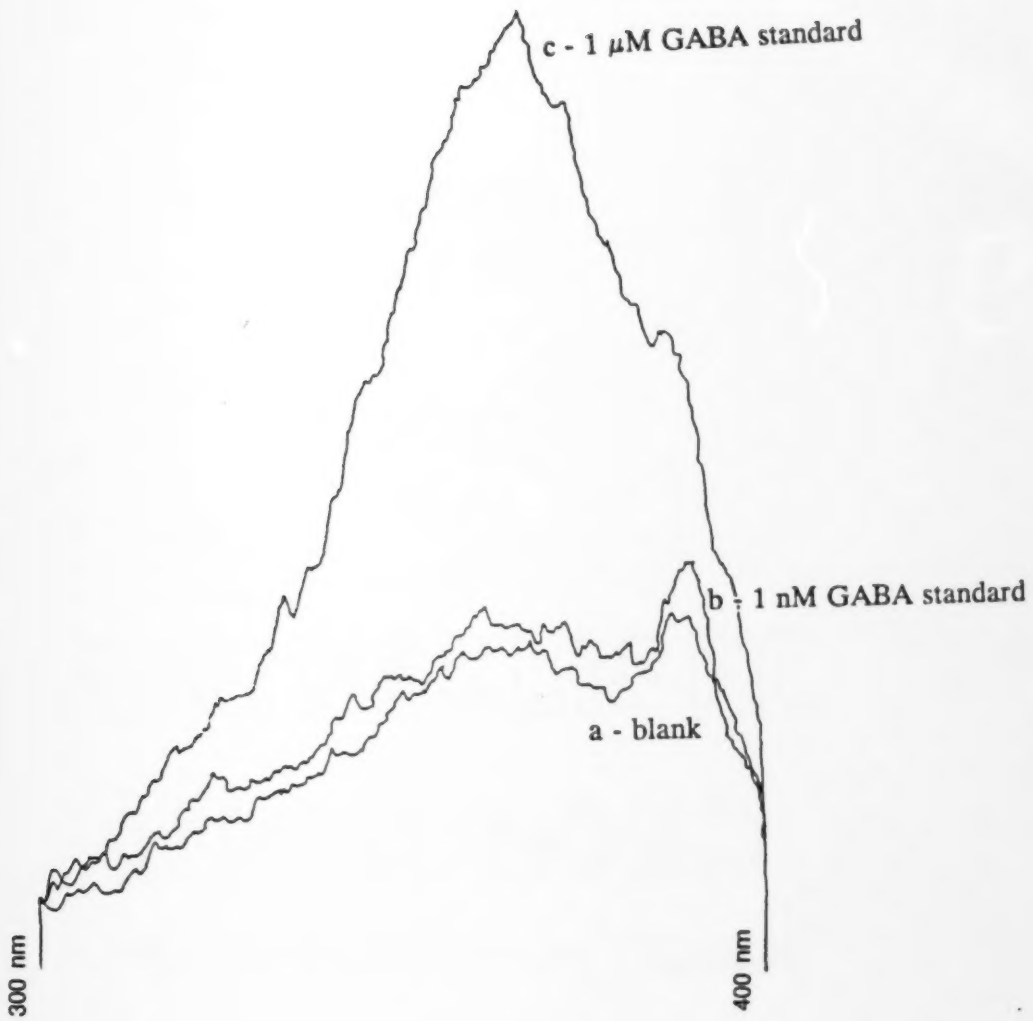


Figure 6.3 Fluorescence spectra from gabase assay. Excitation spectra were recorded from 300 to 400 nm with the emission wavelength set to 460nm. In this example, the upper trace (c) is the signal obtained from a 1μM GABA standard. The 1 nM and "blank" spectra (b and a respectively) are almost identical.

wavelengths of 370nm and 460nm respectively.

RESULTS AND DISCUSSION

Figure 6.3 illustrates the excitation spectra obtained in two preliminary experiments in which different concentrations of GABA were prepared as samples in 25 μL dH_2O. The "blank" trace was recorded with dH_2O only as the sample. Although micromolar concentrations of GABA could be reliably measured it was found that low nanomolar concentrations were more variable. These results indicate that concentrations of less than 10 nM or approximately 0.6 pmoles of GABA could not be accurately measured under the experimental conditions employed.

In other published reports of endogenous GABA release, GABA concentrations are typically analyzed by HPLC (White & Bradford, 1986; McMahon *et.al.*, 1992; Bonnano *et.al.*, 1993). Following stimulation of synaptosomes by high K^+, the synaptosomes are filtered or centrifuged and the perfusate or supernatant is collected. The time resolution of these methods is only 1-10 minutes. When normalized for the stimulation duration and protein concentration, the results from these previous studies would suggest that the amount of GABA released is on the order of 400 pmoles GABA or less per mg of synaptosomal protein per minute. Thus the fluorescence assay using GABase lacks the sensitivity necessary for rapid GABA release studies.

Rapid Mixing and Separation of Synaptosomes and Released Transmitter: As an alternative to continuous fluorescence detection, a rapid mixing technique was investigated as a method to sample transmitter release at brief, rapid intervals following depolarization. If suitable, this method could be used to measure release of radiolabelled neurotransmitters or endogenous transmitters using a sensitive assay such as HPLC (see below).

A rapid mixing device (RMD) which was originally designed to permit rapid mixing of solutions with subsequent collection of mixture samples was obtained from Dr. Ian Martin. This apparatus had been built "in-house" by the technical shop at the

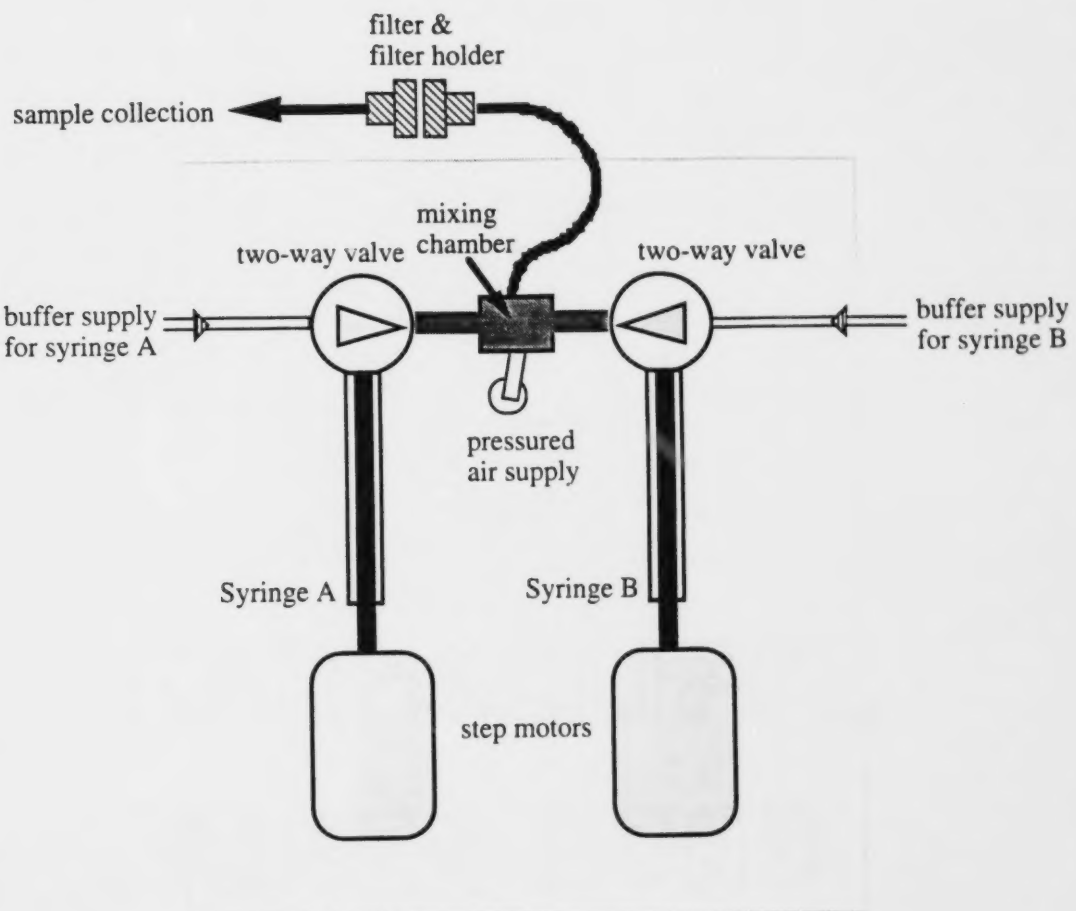


Figure 6.4 Schematic diagram of the Rapid Mixing Device. The two step motors are controlled by a battery power source (not shown). Movement of the motors forces the solutions in the two syringes to mix in the mixing chamber. The mixing chamber is cleared by a pulse of air and the solution is then forced through the filter, that is held in the filter holder, in order to separate the synaptosome vesicles and the perfusate.

University of Cambridge. A schematic drawing of the RMD is shown in Figure 6.4.

The RMD allows mixing of small volumes of two different solutions in a 100 μL teflon mixing chamber. Each solution is initially contained in one of two 1 mL Gastight Hamilton syringes and the volumes that are mixed are controlled independently by step-motors driving each syringe. The speed of each step-motor is also independently controlled. The solutions in the syringes are pushed into a small chamber, and, following a set duration of mixing, the contents of the chamber are cleared by a pulse of compressed air. The period of mixing in the chamber is adjustable; however the duration of the pulse of air is fixed at 10 seconds.

In order for the RMD to be useful for measuring neurotransmitter release, it was necessary to design a filtering system that would allow separation of the synaptosomes from the eluate. The RMD was not originally equipped with this kind of a filtering system. An in-line stainless steel 13 mm filter holder was purchased from Poretics (Livermore, CA), and subsequently, in order to keep the dead volume of the filtering system to a minimum, a 4 mm filter holder was designed and machined by S. Graziano (Technical services, University of Alberta).

Whatman (Maidstone, England) glass fibre filters of types GF/A, GF/B, GF/C and GF/F, as well as cellulose ester filters of .8 μm and .45 μm pore size (Millipore; Bedford, MA) were evaluated in the filter holder, using standard tryptophan solutions, to determine the most suitable based on flow rate, durability and consistency of volume measurements.

RESULTS AND DISCUSSION

The RMD mixes 17 μL of solution from each 1 mL sample syringe with one step of the motor. The first step taken with full syringes was small, probably due to the step motors being slightly misaligned following the filling of the syringes.

With the 4mm filter holder in place, and using cellulose ester filters or GF/F glass fibre filters, the flow rates were inadequate, resulting in problems of back pressure and leaking. GF/B and GF/C provided adequate flow rates but were easily damaged during filtration. Fine wire mesh discs were therefore constructed to provide additional support

for the filters in the holder. It was also observed that the repeatability of the sample volume collected following each shot was quite variable. Even after reducing the length of tubing connecting the mixing chamber and the filter holder, fluid droplets were still evident within the tubing. This residual synaptosome solution from prior mixings would contribute to the variability in volume measurements and would also compromise the time resolution of the system.

A number of components in the RMD were difficult to precisely characterize. The duration of the air pulse could not be adjusted and the pressure of the pulse could not be increased due to limitations of the seals and connections within the RMD. Variability in the actual mechanical function of the step motors was also observed. The settings controlling the timing were not sufficiently precise to determine the exact duration of mixing. Finally it was apparent that logistically, the collection of large numbers of samples would be unwieldy.

HPLC Measurements of Released Amino Acid Transmitters: Since the fluorescence measurements described above lacked the necessary sensitivity or desirable temporal resolution, HPLC was investigated as a sensitive method to quantitate the release of endogenouse neurotransmitters.

Preliminary tests with amino acid standard solutions and synaptosome samples were carried out using a Waters System HPLC consisting of model 510 pumps, a 712 WISP and a model 420/420-AC fluorescence detector.

In initial experiments, synaptosomes were diluted into either non-depolarizing or high K^+ buffer and the extrasynaptosomal fluid (containing released neurotransmitter) was collected by two different methods. A perfusion apparatus with a pump and a Millipore Swinnex filter holder and filter positioned to separate the synaptosomes and perfusate was used. Alternatively, separation of the synaptosomes and buffer containing released neurotransmitter was achieved by centrifuging stimulated and non-stimulated samples through silicone/parafin oil (1.011g/mL density).

In some cases the perfusate or supernatant was extracted with ethanol. An equal volume of 95% ethanol was added to the sample in a microcentrifuge tube. The sample

was pelleted by centrifugation for two minutes and the supernatant was aliquoted into new tubes. These aliquots were dried in a vacuum microcentrifuge. 0.5 mL 75% ethanol was added to each tube and the tubes were centrifuged again. The supernatant was removed, dried and dissolved in 100 μL in dH_2O for HPLC sampling. Amino acid standard samples were prepared from reagent chemical sources in dH_2O as appropriate. For every HPLC run, 50 μL of the given sample was injected onto the column.

Detection of amino acids was achieved with pre-column derivatization with o-phthaldialdehyde (OPA). A solution containing OPA was freshly prepared prior to every HPLC experiment by dissolving 8 mg OPA in 200 μL ethanol. To this solution, 5 mL of borate buffer (0.40 M di-sodium tetraborate, pH=10.4 with NaOH) was added followed by 20 μL β-mercaptoethanol and 30 μL of BRIJ-35 (30% w/v). Addtional borate buffer was added to make the volume up to 10 mLs. This solution was allowed to equilibrate, protected from direct light, for at least 20 minutes.

A reverse-phase octadecyl silane column of particle size 5 μm (C_{18} CSC-ODS2 (Chromatography Sciences Company Inc., SPE Ltd., Concord, Ontario) column was employed. A "guard" column, packed with C18/Corasil 37-50 μm Bondapak (Waters; Milford, MA) was positioned before the main column. The mobile phase contained lithium acetate (pH 5.5) and methanol and a gradient elution protocol was used at a flow rate of 1.5 mL/min. All solutions were degassed by filtering under vacuum. The HPLC was operated at room temperature.

RESULTS AND DISCUSSION

Fluorimetric detection of the OPA-derivatives of amino acid standards was possible with the described system. Figure 6.5 shows a chromatogram for a 100 pmole and a 1 pmole amino acid standard run. Extensive washing of the column and careful preparation of the glassware and solutions did not eliminate all background peaks and control tests with dH_2O indicated a number of "noise" peaks of around 5 pmole.

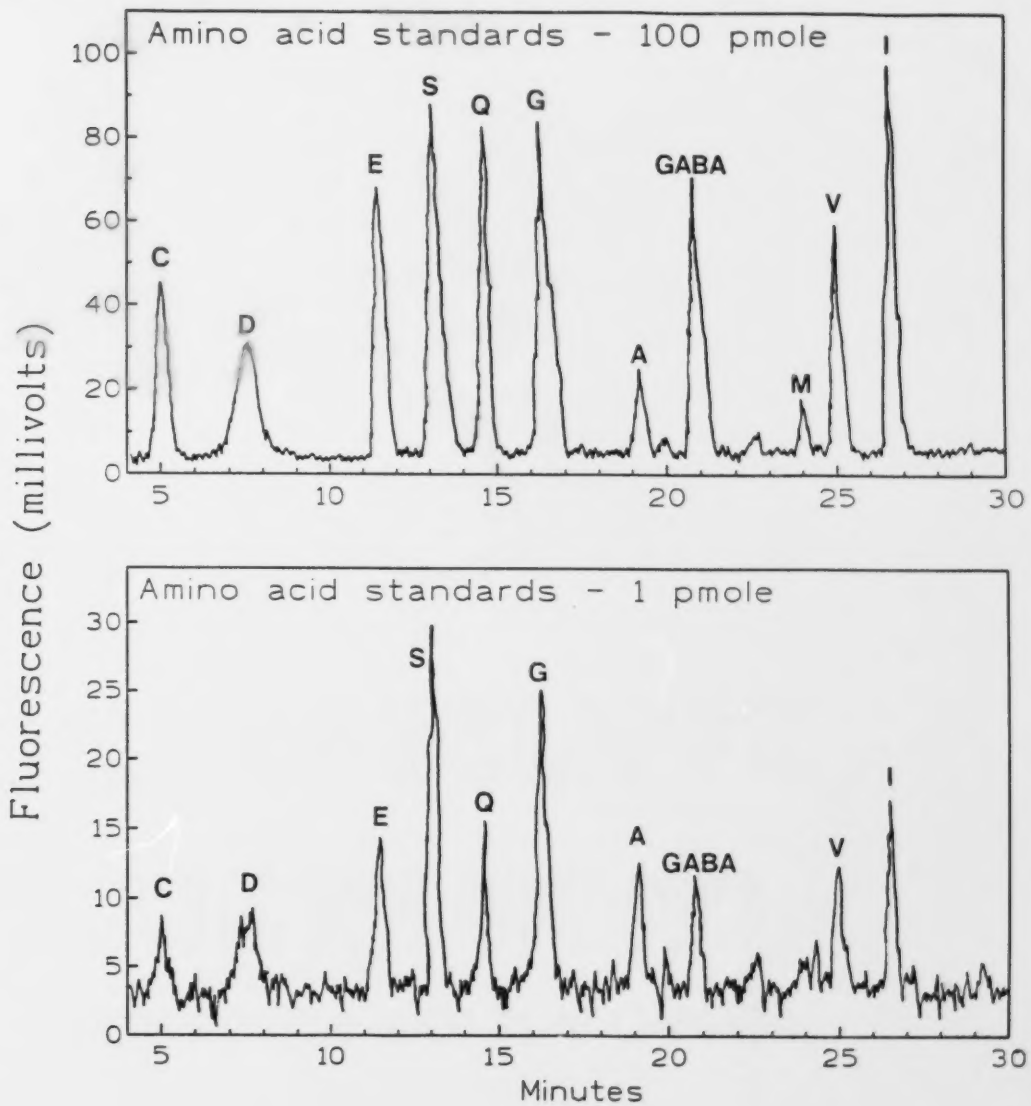


Figure 6.5 HPLC amino acid standard chromatograms. Figures show the fluorescence detected following OPA-derivatization of standard amino acid solutions. The peaks are labelled with the single letter symbol for the amino acid represented (i.e. cysteine (C), aspartate (D), glutamate (E), serine (S), glutamine (Q), glycine (G), alanine (A), methionine (M), norvaline (V), isoleucine (I)).

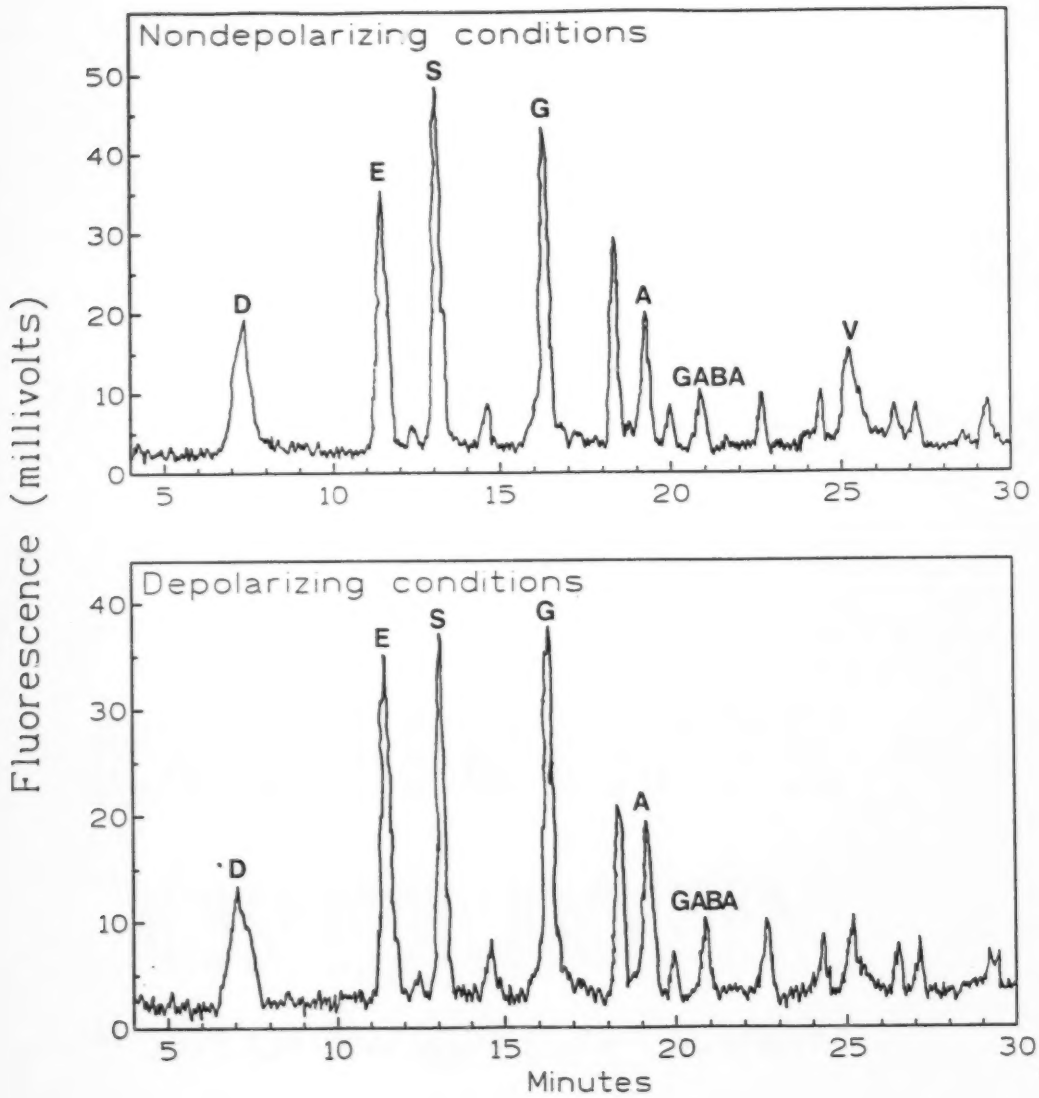


Figure 6.6 HPLC synaptosome perfusate sample chromatograms. Figures show the fluorescence detected following OPA-derivatization of perfusate collected after five minutes of perfusion by either nondepolarizing buffer or by 25 mM KCl buffer. The peaks are labelled with the single letter symbol for the amino acid represented (i.e. aspartate (D), glutamate (E), serine (S), glycine (G), alanine (A), norvaline (V)).

Figure 6.6 shows two chromatograms of samples from synaptosomes that had been perfused with either nondepolarizing choline buffer or choline buffer containing 25 mM KCl. 50 μL of each perfusate was applied to the column. No differences in GABA or glutamate content could be detected between these two samples and the peak area of these two amino acids indicated 9-13 pmole of GABA and 45-46 pmole of glutamate. In several attempts, no consistent differences between the depolarizing and nondepolarizing conditions were observed.

Francis and Lowe (1993) note that the linear range of the fluorescent signal from OPA-derivatives is from 5 to 5000 pmole. The results of control studies with the HPLC system described here would indicate that differences in amino acid content of less than 5-10 pmole could not be reliably measured. The inability to detect differences in the depolarizing and nondepolarizing perfusate or supernatant conditions may reflect either the limited resolution of the detection system or may indicate that the sample preparation techniques employed were inadequate. In view of the desire to obtain samples of transmitter release from synaptosomes on a subsecond timescale and the consequent small sample volumes that would be involved in such a sampling technique, current resolution limits of this HPLC technique preclude its use for this purpose.

It is possible that further efforts to overcome some of the technical difficulties of the RMD would permit this instrument to be used to rapidly sample transmitter release. Although the currently available HPLC system was not sufficiently sensitive, modifications such as using microbore columns and electro-chemical detection (Rowley *et.al.*, 1995) could quite likely increase the sensitivity to the necessary levels. However, the difficulties encountered with the RMD and HPLC systems required another method of measuring neurotransmitter release to be pursued.

Radiolabelled transmitter release: Transmitter release from synaptosomes was measured using a protocol modelled on the method of Henley and co-workers (Barnes *et.al.*, 1994).

Frozen synaptosomes were thawed and diluted to $\approx$ 0.8 mg/mL with Ca^{2+}-free sodium buffer. The diluted synaptosome suspension was divided into three aliquots of

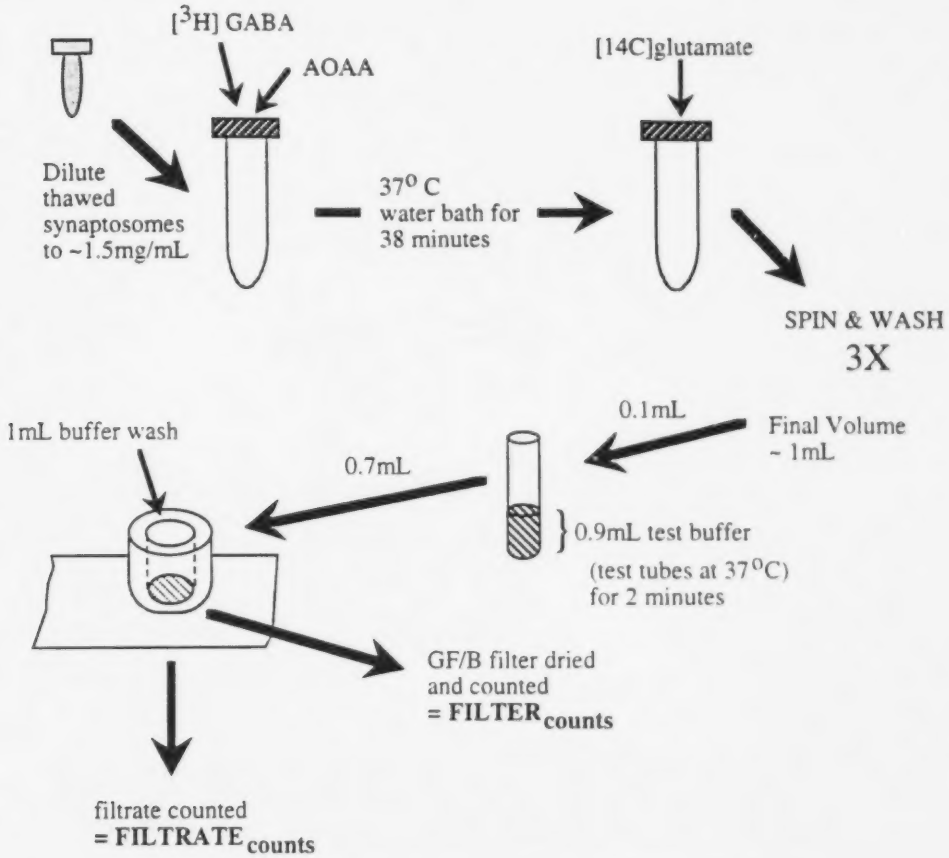
2.5 mLs and all aliquots were kept on ice until the beginning of the incubation with radiolabelled transmitter. The incubations were staggered such that the final incubation began 50 minutes after the synaptosomes were thawed.

The incubation protocol is illustrated in Figure 6.7 For each incubation, 25 μl of freshly prepared 10 mM aminoxyacetic acid (AOAA) and 3 μl of the [^{3}H]GABA stock (1.0 mCi/mL) were added to the 2.5 mL synaptosome aliquot. The solution was vortexed and placed in a 37°C waterbath. The suspension was mixed periodically throughout the incubation. 5 μl of the [^{14}C]glutamate stock (0.1 mCi/mL) was added after 38 minutes and the incubation was continued for an additional 7 minutes.

In most cases, drug or toxin effects on transmitter release were ivestigated by dividing the diluted synaptosomes into 1.5 and 1 mL aliquots and adding the drug to the 1.5 mL aliquot at the beginning of the incubation along with the [^{3}H]GABA or AOAA. In some cases the the [^{3}H]GABA-AOAA-synaptosome solution was divided into two vials at about 20 minutes into the incubation and the drug was added to one of the vials from a stock solution. [^{14}C]glutamate was added as appropriate to all the vials after 38 minutes.

After the 45 minute incubation, the synaptosome mixture(s) was spun down in a desk top centrifuge (45 seconds at the maximum speed $\approx$ 5000 rpm). The supernatants were carefully removed and the pellets were resuspended in nondepolarizing choline buffer, with or without drug as appropriate. The total volume of buffer used in the first wash was 10 mLs. The synaptosome solutions were spun down again and resuspended in a total of 5 mLs of buffer. The solution was spun down a third time and resuspended in a final volume of 500-700 μl buffer.

During the incubation period, ten test tubes were prepared and maintained at 37°C. Each test tube contained 900 μl of depolarizing or nondepolarizing choline buffer, with or without the drug or toxin. 100 μl of the final radiolabelled synaptosome resuspensions were added to each test-tube at 10 second intervals and mixed thoroughly. After 2 minutes, 700 μl of the synaptosome mixture was removed from the first test-tube and applied to a presoaked GF/B filter placed in one well of a filtration apparatus



$$\text{RELEASE} = \frac{\text{FILTRATE}_{\text{counts}}}{\text{total (i.e. FILTER}_{\text{counts}} + \text{FILTRATE}_{\text{counts}})}$$

$$\%\ \text{CHANGE} = \frac{\text{RELEASE}_{\text{condition}} - \text{RELEASE}_{\text{basal}}}{\text{RELEASE}_{\text{KCl}} - \text{RELEASE}_{\text{basal}}}$$

Figure 6.7 Flow chart outlining the protocol used in the dual-labelled neurotransmitter release studies. Uptake of [^{3}H]GABA and [^{14}C]glutamate was allowed to proceed for 45 and 7 minutes, respectively, at 37°C. After a series of centrifugation and wash steps, an aliquot was diluted into a volume of test buffer and filtered under vacuum through a glass fibre filter (GF/B). Filter and filtrate radioactivity were counted and used in the calculations as indicated.

(Hoefer, Model .FH224V). The solution was drawn through the filter by a brief application of vacuum and the filter was washed with 1 mL of nondepolarizing choline buffer with or without the drug/toxin as appropriate. Samples from the remaining test tubes were collected in the same way. The total time for sample collection was typically less than 2.5 minutes.

For each sample, both the filter, after drying under a heat lamp, and filtrate were counted for radioactivity after addition of 5 mL ECOLITE scintillation fluid (ICN, Costa Mesa, CA). The radioactivity was counted in a Beckman model LS5000 scintillation counter using a dual channel program. Standards containing only [^{3}H] or [^{14}C] were included in each experiment in order to account for the contribution of each isotope to the counts recorded in each of the two channels. In addition, the necessary controls for determining and correcting for signal quenching were carried out in each experiment. The data were analysed using the WATFILE/Plus Data Manipulation System (V3.5; WATCOM Systems Inc., Waterloo, Ontario Canada).

The release of [^{3}H]GABA or [^{14}C]glutamate was calculated by dividing the radioactivity due to [^{3}H] or [^{14}C] in the filtrate, by the total radioactivity in the filter and filtrate.

The data of the release of [^{3}H]GABA or [^{14}C]glutamate are presented as a percentage of release stimulated with 25 mM K^+-depolarizing buffer. In each experiment at least two 25 mM K^+-depolarizing and nondepolarizing samples were collected and the difference in the percentage of release in these two conditions was defined as 100% release. This normalization allowed comparison between different experiments.

RESULTS AND DISCUSSION

Loading the Synaptosomes with Radiolabelled Transmitters: [^{3}H]GABA was allowed to equilibrate with the synaptosomes during a 45 minute incubation at 37°C, while the [^{14}C]glutamate was concurrently loaded during the final seven minutes of this incubation. Comparison of the radioactivity in an aliquot of the synaptosome/dual transmitter solution before and after the first centrifugation indicated that 71.6 $\pm$ 0.4 % (n=10) and 61.0 $\pm$ 3.21 % (n=10) was associated with the pellet. Additional radioactivity was removed in

the subsequent washes and centrifugations, but the precise amount of transmitter loaded was not rigorously quantitated.

K^+ and Ca^{2+} Dependence of Neurotransmitter Release: In determining the amount of transmitter released in a given experimental condition, the difference between the percentage of radioactivity released following 25 mM KCl stimulation and nondepolarizing conditions was used as a control. In order to confirm that high K^+ resulted in more radiolabel being released into the filtrate, a two-way analysis of variance (ANOVA) was performed using the average and standard error of the mean (SEM) for all the [^{3}H]GABA and [^{14}C]glutamate release data. The results (Table 6.1) indicate that for both transmitters, the percentage release measured following stimulation by 25 mM KCl was significantly greater ($p < 0.01\%$) than that measured after the loaded synaptosomes were mixed with nondepolarizing buffer. In addition the percentage release was overall significantly greater for [^{14}C]glutamate than for [^{3}H]GABA.

TABLE 6.1

ANOVA Table of Nondepolarizing and 25 mM KCl Depolarizing [^{3}H]GABA and [^{14}C]Glutamate Release

	[^{3}H]GABA		[^{14}C]Glutamate	
CONDITION	MEAN	SEM	MEAN	SEM
Nondepolarizing Buffer	53.2	1.48	62.0	1.13
25 mM KCl Depolarizing Buffer	59.2	1.42	67.0	0.95

n = 33

Main Effect of Condition: $F(1, 128) = 19.11$; $p<0.01\%$

Main Effect of Transmitter: $F(1, 128) = 43.12$; $p<0.01\%$

Interaction Effect: $F(1, 128) = 0.160$; NS

Figure 6.8 shows the results of [^{3}H]GABA and [^{14}C]glutamate release under different conditions of depolarization and in the presence of different calcium concentrations. Release was not reduced when nominally Ca^{2+}-free buffers were used; however with EGTA present to reduce the external calcium concentration to 10 nM, the release of both radiolabelled GABA and glutamate was reduced to approximately 50% of control values. On average, release stimulated by 1 mM 4-AP was only about 40% of the [^{3}H]GABA and [^{14}C]glutamate release stimulated by 25 mM KCl. 15 mM KCl induced release was also less than the control release while increasing the potassium concentration to 50 mM KCl resulted in approximately three times greater release. An analysis of variance and a post-hoc Fisher's LSD test indicated that only the change observed with 50 mM KCl stimulation was statistically significant.

Figure 6.9 shows the effects of several pharmacological agents on the radiolabelled transmitter release. Under the conditions of this study, Cd^{2+} did not block [^{3}H]GABA and [^{14}C]glutamate release. Nitrendipine, nimodipine and ω-conotoxin GVIA appeared to reduce the transmitter release measured. The average block of release observed with 50 nM ω-agatoxin IVA and 100 nM ω-conotoxin MVIIC was quite consistent and the variability of the measurements collected under these conditions were quite small relative to other test conditions. However, the changes observed in the presence of these antagonists were not statistically significant. Thus additional experiments would be required in order to draw firm conclusions as to the effects of calcium channel ligands on neurotransmitter release.

There are some limitations that should be considered in evaluating these results. Using [^{3}H]GABA and [^{14}C]glutamate to label the presynaptic transmitter pools involves the introduction of exogenous compounds into the system. It is difficult to measure how much of the radiolabelled transmitter associates with different compartments within the presynaptic terminal and it is unlikely that equilibration with the Ca^{2+}-dependent releasable pool occurs simultaneously with equilibration across the plasma membrane (Nicholls, 1993). Also inhibiting metabolism by agents such as AOAA can compromise the integrity of the synaptosomes, particularly at higher concentrations (Kauppinen *et.al.*,1987).

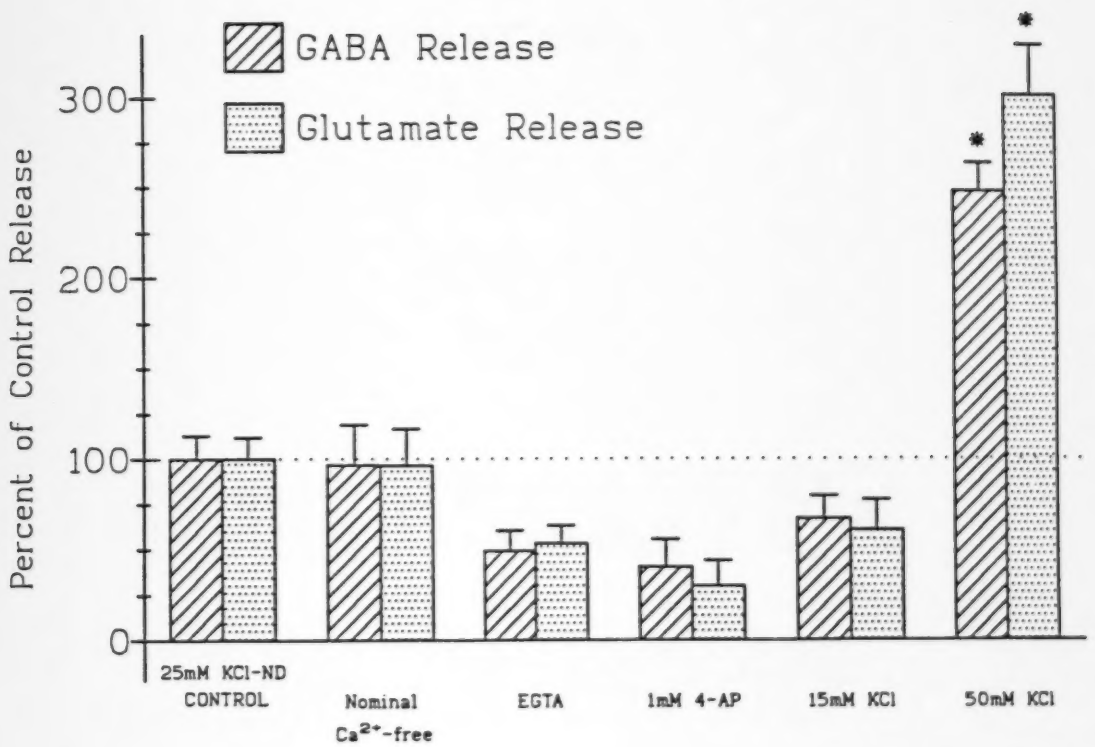


Figure 6.8 [^{3}H]GABA and [^{14}C]glutamate release following depolarization by elevated $[K^+]_o$ or 4-AP and in reduced $[Ca^{2+}]_o$ conditions. The data are presented as the mean and SEM of each experimental condition relative to baseline release measured in 25 mM $[K^+]_o$ and nondepolarizing choline buffers. An "*" indicates a statistically significant difference from baseline release as determined by Fisher's LSD post-hoc test.

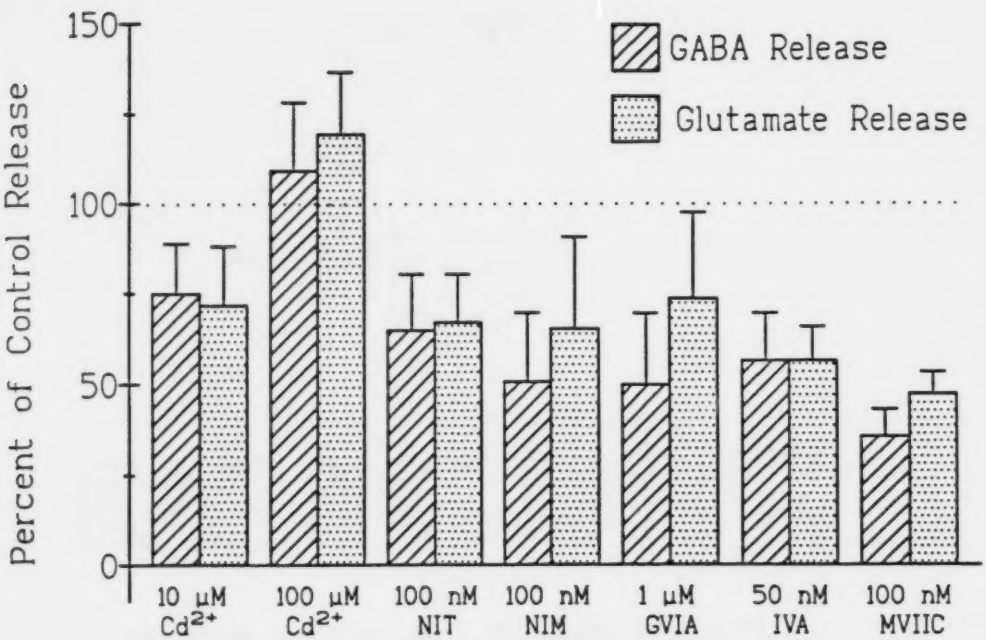


Figure 6.9 [^{3}H]GABA and [^{14}C]glutamate release from synaptosomes incubated in the presence of selected calcium channel ligands. The drugs or toxins were equilibrated with the synaptosomes for at least 20 minutes (37°). The data are presented as the mean and SEM of each experimental condition relative to baseline release measured in 25 mM $[K^+]_o$ and nondepolarizing choline buffers.

During the two minute incubation period employed in this study, reuptake of released transmitter through the plasma membrane transporters would occur. The use of choline rather than sodium buffers in the wash and final incubation media, would tend to reduce this uptake since the activity of the transporter is Na^+-dependent. Choline-substituted buffers would also tend to decrease the amount of Ca^{2+}-independent transmitter release. However, a two minute sustained depolarization is not typical of the physiological situation.

The current protocol permits collection of data at a single time point. Although this approach allows different stimulating conditions to be examined concurrently, it is not suitable for evaluating possible kinetic parameters of the release process as can be done using superfusion or continuous fluorimetric techniques (Nicholls *et.al.*, 1987; Goldin & Turner 1989; Turner *et.al.*, 1995).

A final limitation of the current experimental system is that the practical collection of a given filtrate and filter sample involves several solution transfers after the wash and centrifugation steps. Additional variability is introduced with each step and a more complicated protocol is more difficult to repeat reliablity from experiment to experiment.

SUMMARY OF NEUROTRANSMITTER RELEASE STUDIES

The results of several methods for measuring neurotransmitter release from synaptosomes have been presented and discussed. The ACh luminescence assay was found to be limited in sensitivity. Monitoring the fluorescence of NADPH in order to detect glutamate did not have a time resolution as rapid as desired and a similar enyzme-linked assay for GABA was inadequate due to poor sensitivity. Combining rapid mixing and HPLC to measure amino acid transmitter release in perfusate samples may be a feasible option if certain technical difficulties can be overcome. Variability from experiment to experiment was a problem with [^{3}H]GABA and [^{14}C]glutamate radiolabelled synaptosomes and the time resolution attained using the current protocol was relatively poor. Although none of the methods investigated proved to be ideal, it may be possible to develop an assay to rapidly measure neurotransmitter release by combining some aspects of the different approaches studied here.

Chapter Seven
General Discussion

The purpose of the work undertaken in this thesis was to contribute to the understanding of presynaptic mechanisms regulating Ca^{2+} influx and the release of neurotransmitters in the central nervous system. Biochemical approaches for studying both calcium channel function and transmitter release were developed using rat cortical synaptosomes. The results presented contribute to the fields of pharmacology and neuroscience by furthering our insights into some of the basic aspects of neurotransmission occurring at the level of the nerve terminal.

A stopped-flow fluorescence technique to measure rapid depolarization-induced changes of intrasynaptosomal Ca^{2+} concentrations was developed and used to investigate presynaptic calcium channel function in rat cortical synaptosomes. Rapid flux responses, stimulated by mixing with elevated external K^+ concentrations, are voltage-dependent and blocked by inorganic di- and tri-valent cations. Purified constituents from the venom of the funnel web spider *Agelenopsis aperta*, as well as a peptide toxin from the fish-hunting snail *Conus magus*, ω-conotoxin MVIIC, demonstrated the greatest block of presynaptic Ca^{2+} influx. Depolarization-induced changes in $[Ca^{2+}]_i$ are, however, shown to be relatively insensitive to dihydropyridines and to ω-conotoxin GVIA, a peptide toxin from *Conus geographus*. These pharmacological findings indicate that voltage-dependent calcium channels of the P- and Q-subtypes mediate the majority of presynaptic Ca^{2+} influx following depolarization of the nerve terminal. A number of other pharmacological agents including aminoglycoside antibiotics, neurosteroids and various agonists and antagonists of proposed presynaptic autoreceptors were also examined for an effect on presynaptic Ca^{2+} influx; however the results obtained indicate that these compounds do not generally change the characteristics of the Ca^{2+} influx as measured by stopped-flow fluorimetry under the studied conditions.

Different depolarizing protocols were used to investigate whether any pharmacological features of the influx of Ca^{2+} through the channels would demonstrate sensitivity to the degree and type of depolarization induced in the synaptosome

preparation. Although the greatest inhibition of Ca^{2+} influx was observed with the peptide toxins ω-conotoxin MVIIC and ω-agatoxin IVA in both K^+ and 4-AP depolarizing protocols, the latter method of depolarization generated a Ca^{2+} influx that was slightly more sensitive to 1,4-dihydropyridines and ω-conotoxin GVIA. These findings indicate the potential of rapid methods of detecting presynaptic Ca^{2+} fluxes, combined with the considered use of specific pharmacological tools to regulate membrane excitability, to study the details of presynaptic calcium channel activity.

Immunoglobulins were purified from individuals with amyotrophic lateral sclerosis and tested with Fura-2 loaded synaptosomes in order to further investigate the possibility that autoimmunity to presynaptic calcium channels is involved in the pathogenesis of this neurodegenerative disease. The results obtained with the eight ALS samples did not suggest that presynaptic Ca^{2+} influx was modified. ALS is a complex disorder and it is likely that many factors contribute to the disease process.

In order to determine if a correlation between the pharmacology of the Ca^{2+} influx observed and release of transmitter could be established, the development of a rapid assay for the measurement of transmitter release was attempted. Three approaches were considered. In the interest of utilizing the advantages of fluorescence and the stopped-flow fluorimetric technique, a luminescent assay for measuring acetylcholine (Israel & Lesbats, 1981) and a fluorimetric assay for glutamate (Nicholls *et.al.*, 1987) were evaluated. These assays were found to lack either sufficient sensitivity or the desired time resolution and were not pursued extensively. A system employing an in-house built apparatus that permitted mixing of the synaptosomes and buffers with a subsequent separation of vesicle and perfusate appeared promising as a technique with more rapid time resolution of endogenous transmitter release. A fluorimetric assay for the detection of GABA in the perfusate samples was investigated but found to lack the necessary sensitivity for detection of this amino acid. High performance liquid chromatography was also considered for analyzing the perfusate for amino acid transmitters. Many aspects of this method were evaluated; however technical difficulties with the design of the mixing apparatus, as well as the extreme demands on HPLC sensitivity given the low sample volumes, required an alternate approach to be taken.

The third technique for measuring neurotransmitter release involved loading the synaptosomes with radiolabelled GABA and glutamate and monitoring the release of the transmitters following vacuum filtration, by scintillation counting. Pre-loading synaptosomes with two transmitters was a novel aspect of this approach. Although the results reported herein are preliminary, dual-labelling of the synaptosomes with [^{3}H]GABA and [^{14}G]glutamate appears to be feasible. [^{3}H]GABA and [^{14}G]glutamate release was shown to be dependent on the degree of depolarization induced. The release of [^{3}H]GABA or [^{14}G]glutamate was not differentially affected by any of the drugs or toxins tested. Modification of this method in order to increase the time resolution is desirable, and a combination of loading the synaptosomes with labelled transmitter and using the RMD for separation and rapid collection of samples may be a workable choice for pursuing this aspect of the project.

Synaptosomes are a versatile preparation for studies of presynaptic mechanisms; however the heterogenity of the nerve terminals is a limitation. In the current work, the Ca^{2+} signal recorded on depolarization was shown to be sensitive to inhibition by a variety of antagonists of calcium channels. Whether all the teminals contain all subtypes of channel, or if there is a differential distribution across synaptosomes, however, can not be determined. In addition, the use of Fura-2 provides a spatially and temporally averaged indication of the change in intrasynaptosomal Ca^{2+} levels. It is not possible to extrapolate from such a signal the exact contribution of some specific source of Ca^{2+} because many factors contribute to produce the actual fluorescence signal. Nevertheless, synaptosomes can be productively employed, with consideration of these limitations, to provide insights into presynaptic mechanisms unattainable by any other experimental method.

The results of the studies reported here support the use of synaptosomes and stopped-flow fluorimetry as a versatile and effective method for investigating presynaptic mechanisms of calcium channel function and transmitter release. The development of this stopped-flow fluorimetric technique to monitor presynaptic calcium fluxes with sub-second time resolution is a significant advance. Combining such rapid kinetic measurements of Ca^{2+} influx with rapid methods of measuring neurotransmitter release

will undoubtedly continue to contribute to our understanding of presynaptic mechanisms of neurotransmission.

GENERAL REFERENCES

Adamson P., Hajimohamadreza, Brammer M.J. and Campbell I.C., (1989) Intrasynaptosomal free calcium concentration is increased by phorbol esters via a 1,4-dihydropyridine-sensitive (L-type) Ca^{2+} channel, *Eur. J. Pharmacol.*, **162**:59-66.

Agoston D., Hargittai P. and Nagy A., (1983) Effects of a 4-aminopyridine in calcium movements and changes of membrane potential in pinched-off nerve terminals from rat cerebral cortex, *J. Neurochem.*, **41**:745-751.

Ahlijanian, M.K., Westenbroek, R.E. and Catterall, W.A., (1990) Subunit structure and localization of dihydropyridine-sensitive calcium channels in mammalian brain, spinal cord, and retina, *Neuron*, **4**:819-832.

Almers W., (1994) How fast can you get?, *Nature*, **367**:682-683.

Antel J.P. and Cashman N.R., (1995) Immunological findings in amyotrophic lateral sclerosis, *Springer Semin. Immunopathol.*, **17**:17-28.

Appel S.H., Engelhardt J.I., García J. and Stefani E., (1991) Immunoglobulins from animal models of motor neuron disease and from human amyotrophic lateral sclerosis patients passively transfer physiological abnormalities to the neuromuscular junction, *Proc. Natl. Acad. Sci. U.S.A.*, **88**:647-651.

Appel S.H., Smith R.G., Engelhardt J.I. and Stefani E., (1993) Evidence for autoimmunity in amyotrophic lateral sclerosis, *J. Neurological Sci.*, **118**:169-174.

Appel S.H. and Stefani E., (1991) Amyotrophic lateral sclerosis: etiology and pathogenesis, *Curr. Neurology*, **11**:289-310.

Atchison W.D., Adgate L. and Beaman C.M., (1988) Effects of antibiotics on uptake of calcium into isolated nerve terminals, *J. Pharmacol. Exp. Ther.*, **245**:394-401.

Augustine G.J., Adler E.M. and Charlton M.P., (1991) The calcium signal for transmitter secretion from presynaptic nerve terminals, *Ann. NY Acad. Sci.*, **635**:365-381.

Barhanin, J., Schmid, A. and Lazdunski, M., (1988) Properties of structure and interaction of the receptor for ω-conotoxin, a polypeptide active on Ca^{2+} channels, *Biochem. Biophys. Res. Commun.*, **150**:1051-1062.

Barnes J.M., Kumlesh K.D. and Henley J.M., (1994) Cyclothiazide unmasks AMPA-evoked stimulation of [^{3}H]-L-glutamate release from rat hippocampal synaptosomes, *Brit. J. Pharmacol.*, **113**:339-341.

Bartschat D.K. and Blaustein M.P., (1985) Potassium channels in isolated presynaptic nerve terminals from rat brain, *J. Physiol. (Lond.)*, **361**:419-440.

Bartschat D.K. and Rhodes T.E., (1995) Protein kinase C modulates calcium channels in isolated presynaptic nerve terminals of rat hippocampus, *J. Neurochem.*, **64**:2064-2072.

Bennett M.K. and Scheller R.H., (1994) A molecular description of synaptic vesicle membrane trafficking, *Ann. Rev. Biochem.*, **63**:63-100.

Bernath S., (1992) Calcium-independent release of amino acid neurotransmitters: fact or artifact?, *Prog. Neurobiol.* **38**:57-91.

Berthold F. and Maly F-E., (1987) Instrumentation for cellular chemiluminescence, In: *Cellular Chemiluminescence, Vol. 1.* pp. 49-57. (Eds) K. Van Dyke and V. Castranova. CRC Press Inc.: USA.

Bertolino M. and Llinás, R.R., (1992) The central role of voltage-activated and receptor-operated calcium channels in neuronal cells, *Ann. Rev. Pharmacol. Toxicol.*, **32**:399-421.

Birnbaumer L., Campbell K.P., Catterall W.A., Harpold M.M., Hofmann F., Horne W.A., Mori Y., Schwartz A., Snutch T.P., Tanabe T. and Tsien R.W., (1994) Matters arising: the naming of voltage-gated calcium channels, *Neuron*, **13**:505-506.

Blaustein, M.P. and Goldring, J.M., (1975) Membrane potentials in pinched-off presynaptic nerve terminals monitored with a fluorescent probe: evidence that synaptosomes have potassium diffusion potentials, *J. Physiol. (Lond.)*, **247**:589-615.

Bonanno G., Pittaluga A., Fedele E., Fontana G. and Raiteri M., (1993) Glutamic acid and γ-aminobutyric acid modulate each other's release through heterocarriers sited on the axon terminals of rat brain, *J. Neurochem.*, **61**:222-230.

Brazier M.A.B., (1988) A History of Neurophysiology in the 19th Century, Raven Press, New York.

Brown A.M., (1993) Membrane-delimited cell signaling complexes: direct ion channel regulation by G proteins, *J. Memb. Biol.*, **131**:93-104.

Canzoniero L.M.T., Taglialatela M., Di Renzo G. and Annunziato L., (1993) Gadolinium and neomycin block voltage-sensitive Ca^{2+} channels without interfering with the Na^{+}-Ca^{2+} antiporter in brain nerve endings, *Eur. J. Pharmacol.*, **245**:97-103.

Carbone, E. and Swandulla, D., (1989) Neuronal calcium channels: kinetics, blockade and modulation, *Prog. Biophys. Molec. Biol.*, **54**:31-58.

Carvalho C.M., Ferreira I.L., Duarte C.B., Malva J.O., Tretter L., Adam-Vizi V. and Carvalho A.P. (1995) Relation of $[Ca^{2+}]_i$ to dopamine release in striatal synaptosomes: role of Ca^{2+} channels, *Brain Res.*, **669**:234-244.

Catterall W.A., (1995) Structure and function of voltage-gated ion channels, *Ann. Rev. Biochem.*, **64**:493-531.

Chapell R. and Rosenberg P., (1992) Specificity of action of β-bungarotoxin on acetylcholine release from synaptosomes, *Toxicon*, **30**:621-33.

Cherksey, B.D., Sugimori, M. and Llinás, R.R., (1991) Properties of calcium channels isolated with spider toxin, FTX, *Ann. NY Acad. Sci.*, **635**:80-89.

Coffey E.T., Sihra T.S. and Nicholls D.G., (1993) Protein kinase C and the regulation of glutamate exocytosis from cerebrocortical synaptosomes, *J. Biol. Chem.*, **268**:21060-21065.

Dagani, F. and Erecińska, M., (1987) Relationships among ATP synthesis, K^{+} gradients, and neurotransmitter amino acid levels in isolated rat brain synaptosomes, *J. Neurochem.*, **49**:1229-1240.

Dale H., (1937) Transmission of nervous effects by acetylcholine, *Harvey Lectures*, **32**:229-245.

Delbono O., García J., Appel S.H. and Stefani E., (1991) IgG from amyotrophic lateral sclerosis affects tubular calcium channels of skeletal muscle, *Am. J. Physiol.*, **260**:C1347-C1351.

Deutsch, C., Drown, C., Rafalowska, U. and Silver, I.A., (1981) Synaptosomes from rat brain: morphology, compartmentation, and transmembrane pH and electrical gradients, *J. Neurochem.*, **36**:2063-2072.

Deutsch C. and Rafalowska U., (1979) Transmembrane electrical potential measurements in rat brain synaptosomes, *FEBS Letters*, **108**:274-278.

De Waard M., Witcher D.R. and Campbell K.P., (1994) Functional properties of the purified N-type Ca^{2+} channel from rabbit brain, *J. Biol. Chem.*, **269**:6716-6724.

Diebler M.F., (1982) Acetylcholine release from isolated synaptic vesicles related to ionic permeability changes: continuous detection with a chemiluminescent method, *J. Neurochem.*, **39**:1405-1411.

Dolphin A.C., (1990) G protein modulation of calcium currents in neurons, *Ann. Rev. Physiol.*, **52**:243-255.

Dunlap K., Luebke J.I. and Turner T.J., (1994) Identification of calcium channels that control neurosecretion, *Science*, **266**:828-829.

Dunn, S.M.J., (1988) Multiple calcium channels in synaptosomes: voltage dependence of 1,4-dihydropyridine binding and effects on function, *Biochemistry*, **27**:5275-5281.

Duval D., Durant S. and Homo-Delarche F., (1983) Non-genomic effects of steriods: interactions of steroid molecules with membrane structures and functions, *Biochim. Biophys. Acta*, **737**:409-442.

Eccles J.C., (1946) An electrical hypothesis of synaptic and neuro-muscular transmission, *Ann. NY Acad. Sci.*, **47**:429-455.

Eccles J.C., (1975) Under the spell of the synapse, In: *The Neurosciences: Paths of Discovery*. pp. 159-179. Eds. F.G. Worden, J.P. Swazey and G. Adelman. Colonial Press Inc.:Cambridge MA.

Eisen A., (1995) Amyotrophic lateral sclerosis is a multifactorial disease, *Muscle & Nerve*, **18**:741-752.

Elferink L.A., Peterson M.R. and Scheller R.H., (1993) A role for synaptotagmin (p65) in regulated exocytosis, *Cell*, **72**:153-159.

Engelhardt J.I., Appel S.H. and Killian J.M., (1990) Motor neuron destruction in guinea pigs immunized with bovine spinal cord ventral horn homogenate: experimental autoimmune gray matter disease, *J. Neuroimmunol.*, **27**:21-31.

Erecińska, M., Nelson D., Yudkoff M. and Silver I.A., (1994) Energetics of the nerve terminal in relation to central nervous system function, *Biochem. Soc. Trans.*, *22:959-965.*

Fanelli R.J., McCarthy R.T. and Chisholm J., (1994) Neuropharmacology of nimodipine: from single channels to behavior, *Ann. NY Acad. Sci.*, **747**:336-350.

ffrench-Mullen J.M.H. and Spence K.T., (1991) Neurosteroids block Ca^{2+} channel current in freshly isolated hippocampal CA1 neurons, *Eur. J. Pharmacol.*, **202**:269-272.

Fox A.P., Hirning L.D., Mogul D.J., Artalejo C.R., Penington N.J. Scroggs R.S. and Miller R.J., (1991) Modulation of calcium channels by neurotransmitters, hormones and second messengers, In: *Calcium Channels: Their Properties, Functions, Regulation and Clinical Relevance.* pp. 251-263. Eds. L. Hurwitz, L.D. Partridge and J.K. Leach. CRC Press: Boca Raton FL, USA.

Francis P.T. and Lowe S.L., (1993) Determination of transmitter- and non-transmitter-related amino acids in human samples by high performance liquid chromatography, In: *High Performance Liquid Chromatography in Neuroscience Research.* pp. 117-138. Eds. R.B. Holman and A.J. Cross. John Wiley & Sons Ltd.: West Sussex, UK.

Fried G., (1995) Synaptic vesicles and release of transmitters: new insights at the molecular level, *Acta Physiol. Scand.*, **154**:1-15.

Geppert M., Goda Y., Hammer R.E., Li C., Rosahl T.W., Stevens C.F. and Südhof T.C., (1994) Synaptotagmin I: a major Ca^{2+} sensor for transmitter release at a central synapse, *Cell*, **79**:717-727.

Gordon-Weeks P.R., (1987) Isolation of synaptosomes, growth cones and their subsellular components, In: *Neurochemistry: A Practical Approach.* pp. 1-26. Eds A.J. Turner and H.S. Bachelard. IRL Press: Washington DC.

Gotow T., (1992) The cytoplasmic structure of the axon terminal, *Prog. Neurobiol.*, **39**:443-474.

Grassi C., Magnelli V., Carabelli V., Sher E. and Carbone E., (1994) Inhibition of low- and high-threshold Ca^{2+} channels of human neuroblastoma IMR32 cells by Lambert-Eaton myasthenic syndrome (LEMS) IgGs, *Neurosci. Letts.*, **181**:50-56.

Greengard P., Valtorta F., Czernik A.J. and Benfenati F., (1993) Synaptic vesicle phosphoproteins and regulation of synaptic function, *Science*, **259**:780-785.

Grynkiewicz, G., Poenie, M. and Tsien, R.Y., (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties, *J. Biol. Chem.*, **260**: 3440-3450.

Heemskerk F.M.J., Schrama L.H., Ghijsen W.E.J.M., De Graan P.N.E., Lopes da Silva F.H. and Gispen W.H., (1991) Presynaptic mechanism of action of 4-aminopyridine: changes in intracellular free Ca^{2+} concentration and its relationship to B-50 (GAP-43) phosphorylation, *J. Neurochem.*, **56**:1827-1835.

Herrero I., Castro E., Miras-Portugal M.T. and Sánchez-Prieto J., (1991) Glutamate exocytosis evoked by 4-aminopyridine is inhibited by free fatty acids released from rat cerebrocortical synaptosomes, *Neurosci. Letters*, **126**:41-44.

Herrero I., Miras-Portugal M.T. and Sánchez-Prieto J., (1992a) Activation of protein kinase C by phorbol esters and arachidonic acid required for the optimal potentiation of glutamate exocytosis, *J. Neurochem.*, **59**:1574-1577.

Herrero I., Miras-Portugal M.T. and Sánchez-Prieto J., (1992b) Positive feedback of glutamate exocytosis by metabotropic presynaptic receptor stimulation, *Nature*, **360**:163-166.

Hewett S.J. and Atchison W.D., (1992) Specificity of Lambert-Eaton Myasthenic Syndrome immunoglobulin for nerve terminal calcium channels, *Brain Res.*, **599**:324-332.

Hille B., (1992) *Ionic Channels of Excitable Membranes*, Sunderland, MA, Sinauer.

Hillyard, D.R., Monje, V.D., Mintz, I.M., Bean, B.P., Nadasdi, L., Ramachandran, J., Miljanich, G., Azimi-Zoonooz, A., McIntosh, J.M., Cruz, L.J., Imperial, J.S. and Olivera, B.M., (1992) A new conus peptide ligand for mammalian presynaptic Ca^{2+} channels, *Neuron*, **9**:69-77.

Hinkle P.M., Shanshala II E.D. and Nelson E.J., (1992) Measurement of intracellular cadmium with fluorescent dyes, *J. Biol. Chem.*, **267**:25553-25559.

Hirsch H.E. and Robins E., (1962) Distribution of γ-aminobutyric acid in the layers of the cerebral and cerebellar cortex. Implications for its physiological role, *J. Neurochem.*, **9**:63-70.

Hodgkin A.L. and Huxley A.F., (1952) A quantitative description of membrane current and its application to conduction and excitation in nerve, *J. Physiol. (Lond.)*, **117**:500-544.

Hodgkin A.L. and Keynes R.D., (1957) Movements of labelled calcium in squid giant axon, *J. Physiol. (Lond.)*, **138**:253-281.

Hodgkin A.L. and Keynes R.D., (1957) Movements of labelled calcium in squid giant axon, *J. Physiol. (Lond.)*, **138**:253-281.

Hofmann F., Biel M. and Flockerzi V., (1994) Molecular basis for Ca^{2+} channel diversity, *Ann. Rev. Neurosci.*, **17**:399-418.

Hu P.-S., (1993) On the usefulnesss of Fura-2 measurements of intrasynaptosomal calcium levels in rat cortical synaptosomes to study mechanisms of presynaptic function, *Acta Physiol. Scand.*, **148**:115-123.

Isom L.L., De Jongh K.S. and Catterall W.A., (1994) Auxiliary subunits of voltage-gated ion channels, *Neuron*, **12**:1183-1194.

Israel M. and Lesbats B., (1981) Chemiluminescent determination of acetylcholine and continuous detection of its release from *Torpedo* electric organ synapses and synaptosomes, *Neurochem. Int.*, **3**:81-90.

Jahn R. and Südhof T.C., (1994) Synaptic vesicles and exocytosis, *Ann. Rev. Neurosci.*, **17**:219-246.

Jan L.Y. and Jan Y.N., (1989) Voltage-sensitive ion channels, *Cell*, **56**:13-25.

Janis R.A. and Triggle D.J., (1991) Drugs acting on calcium channels, In: *Calcium Channels: Their Properties, Functions, Regulation and Clinical Relevance.* pp. 195-249. Eds. L.Hurwitz, L.D. Partridge and J.K. Leach. CRC Press: Boca Raton FL, USA.

Katz B. and Miledi R., (1968) The role of calcium in neuromuscular facilitation, *J. Physiol. (Lond.)*, **195**:481-492.

Kauppinen R.A., Sihra T.S. and Nicholls D.G., (1987) Aminooxyacetic acid inhibits the malate-aspartate shuttle in isolated nerve terminals and prevents the mitochondria from utilizing glycolytic substrates, *Biochim. Biophys. Acta*, **930**:173-178.

Kauppinen R.A., (1994) From the synaptosome to the intact brain, *Biochem. Soc. Trans.*, **22**:965-969.

Khachaturian Z.S., (1994) Calcium hypothesis of Alzheimer's disease and brain aging, *Ann. NY Acad. Sci.*, **747**:1-11

Kimura F., Smith R.G., Delbono O., Nyormoi O., Schneider T., Nastainczyk W., Hofmann F., Stefani E. and Appel S.H., (1994) Amyotrophic lateral sclerosis patient antibodies label Ca^{2+} channel α_1 subunit, *Ann. Neurology*, **35**:164-171.

Kish P.E. and Ueda T., (1989) Glutamate accumulation into synaptic vesicles, *Meth. Enzymol.*, **174**:9-25.

Knaus H.-G., Striessnig J., Koza A. and Glossman H., (1987) Neurotoxic aminoglycoside antibiotics are potent inhibitors of [^{125}I]-Omega-Conotoxin GVIA binding to guinea-pig cerebral cortex membranes, *Nauyn-Schmiedeberg's Arch. Pharmacol.*, **336**:583-586.

Komulainen, H. and Bondy, S.C., (1987) The estimation of free calcium within synaptosomes and mitochondria with fura-2; comparison to quin-2, *Neurochem. Int.*, **10**:55-64.

Laemmli U.K., (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature*, **227**:680-685.

Lai Y., Peterson B.Z. and Catterall W.A., (1993) Selective dephosphorylation of the subunits of skeletal muscle calcium channels by purified phosphoprotein phosphatases, *J. Neurochem.*, **61**:1333-1339.

Lentzner, A., Bykov, V. and Bartschat, D.K., (1992) Time-resolved changes in intracellular calcium following depolarization of rat brain synaptosomes, *J. Physiol. (Lond.)*, **450**:613-628.

Llinás, R., Sugimori, M., Cherksey, B.D., Smith R.G., Delbono, O., Stefani, E. and Appel, S., (1993) IgG from amyotrophic lateral sclerosis patients increases current through P-type calcium channels in mammalian cerebellar Purkinje cells and in isolated channel protein in lipid bilayer, *Proc. Natl. Acad. Sci. U.S.A.*, **90**:11743-11747.

Llinás R., Sugimori M., Hillman D.E. and Cherksey B., (1992) Distribution and functional signficance of the P-type, voltage-dependent Ca^{2+} channels in the mammalian central nervous system, *Trends Neurosci.*, **15**:351-355.

Llinás, R., Sugimori, M., Lin, J-W. and Cherksey, B., (1989) Blocking and isolation of a calcium channel from neurons in mammals and cephalopods utilizing a toxin fraction (FTX) from funnel-web spider poison, *Proc. Natl. Acad. Sci. U.S.A.*, **86**:1689-1693.

Lowry O.H., Roberts N.R. and Kapphahn J.I., (1957) The fluorometric measurement of pyridine nucleotides, *J. Biol. Chem.*, **224**:1047-64.

Lundy, P.M., Hong, A. and Frew, R., (1992) Inhibition of a dihydropyridine, ω-conotoxin insensitive Ca^{2+} channel in rat synaptosomes by venom of the spider *Hololena curta*, *Eur. J. Pharamcol.*, **225**:51-56.

Lundy, P.M., Frew, R., Fuller, T.W. and Hamilton, M.G., (1991) Pharmacological evidence for an ω-conotoxin, dihydropyridine-insensitive neuronal Ca^{2+} channel, *Eur. J. Pharamcol.*, **206**:61-68.

Lundy, P.M., Stauderman K., Goulet J.-C. and Frew, R., (1989) Effect of ω-conotoxin GVIA on Ca^{2+} influx and endogenous ACh release from chicken brain preparations, *Neurochem. Int.*, **14**:49-55.

Magnelli V., Sawada T., Delbono O., Smith R.G., Appel S.H. and Stefani E., (1993) The action of amyotrophic lateral sclerosis immunoglobulins on mammalian single skeletal muscle Ca^{2+} channels, *J. Physiol. (Lond.)*, **461**:103-118.

Maubecin V.A., Sanchez V.N., Rosato Siri M.D., Cherksey B.D., Sugimori M., Llinás R. and Uchitel O.D. (1995) Pharmacological characterization of the voltage-dependent Ca^{2+} channels present in synaptosomes from rat and chicken central nervous system, *J. Neurochem.*, **64**:2544-2551.

McCarthy R.T. and TanPiengco P.E., (1992) Multiple types of high-threshold calcium channels in rabbit sensory neurons: high-affinity block of neuronal L-type by nimodipine, *J. Neurosci.*, **12**:2225-2234.

McCleskey E.W., (1994) Calcium channels: cellular roles and molecular mechanisms, *Curr. Opin. Neurobiology*, **4**:304-312.

McMahon H.T., Foran P., Dolly J.O., Verhage M., Weigant V.M. and Nicholls D.G., (1992) Tetanus toxin and boutulinum toxins type A and B inhibit glutamate, γ-aminobutyric acid, aspartate and met-enkephalin release from synaptosomes, *J. Biol. Chem.*, **267**:21338-21343.

McMahon, H.T. and Nicholls, D.G., (1991a) The bioenergetics of neurotransmitter release, *Biochim. Biophys. Acta*, **1059**:243-264.

McMahon H.T. and Nicholls D.G., (1991b) Transmitter glutamate release from isolated nerve terminals: evidence for biphasic release and triggering by localized Ca^{2+}, *J. Neurochem.*, **56**:86-94.

Middlemiss D.N. and Spedding M., (1985) A functional correlate for the dihydropyridine binding site in the rat brain, *Nature*, **314**:94-96.

Miljanich G.P. and Ramachandran J., (1995) Antagonists of neuronal calcium channels: structure, function and therapeutic implications, *Ann. Rev. Pharmacol. Toxicol.*, **35**:707-734.

Miller, R.J. and Fox, A.P., (1990) Voltage sensitive calcium channels. In: *Intracellular calcium regulation.* pp.96-138. Ed. F. Bronner. Wiley-Liss Inc.: New York.

Mintz, I.M., Venema, V.J., Swiderek, K.M., Lee, T.D., Bean, B.P. and Adams, M.E., (1992) P-type calcium channels blocked by the spider toxin ω-Aga-IVA, *Nature*, **335**:827-829.

Nachmansohn D., (1946) Chemical mechanism of nerve transmission, *Ann. NY Acad. Sci.*, **47**:395-427.

Nachshen, D.A., (1984) Selectivity of the Ca binding site in synaptosome Ca channels: inhibition of Ca influx by multivalent metal cations, *J. Gen. Physiol.*, 83:941-967.

Neher E., (1995) The use of Fura-2 for estimating Ca buffers and Ca fluxes, *Neuropharmacology*, **34**:1423-1442.

Nicholls, D.G., (1989) Release of glutamate, aspartate, and γ-aminobutyric acid from isolated nerve terminals, *J. Neurochem.*, **52**:331-341.

Nicholls D.G., (1993) The glutamatergic nerve terminal, *Eur. J. Biochem.*, **212**:613-631.

Nicholls D.G. and Sihra T.S., (1986) Synaptosomes possess an exocytotic pool of glutamate, *Nature* **321**:772-773.

Nicholls D.G., Sihra T.S. and Sanchez-Prieto J., (1987) Calcium-dependent and -independent release of glutamate from synaptosomes monitored by continuous fluorometry, *J. Neurochem.*, **50**:50-57.

Nichols R.A., Suplick G.R. and Brown J.M., (1994) Calcineurin-mediated protein dephosphorylation in brain nerve terminals regulates the release of glutamate, *J. Biol. Chem.*, **269**:23817-23823.

Olivera, B.M., Miljanich G.P., Ramachandran J. and Adams, M.E., (1994) Calcium channel diversity and neurotransmitter release: the ω-conotoxins and ω-agatoxins, *Ann. Rev. Biochem.*, **63**:823-867.

Perrier, M.L., Scatton, B. and Benavides, J., (1992) Dihydropyridine- and ω-conotoxin-resistant, neomycin-sensitive calcium channels mediate the depolarization-induced increase in internal calcium levels in cortical slices from immature rat brain, *J. Pharmacol. Exp. Ther.*, **261**:324-330.

Pocock, J.M., and Nicholls, D.G., (1992) A toxin (Aga-GI) from the venom of the spider *Agelenopsis aperta* inhibits the mammalian presynaptic Ca^{2+} channel coupled to glutamate exocytosis, *Eur. J. Pharmacol.*, **226**:343-350.

Ray, R., Morrow, C.S. and Catterall, W.A., (1978) Binding of scorpion toxin to receptor sites associated with voltage-sensitive sodium channels in synaptic nerve ending particles, *J. Biol. Chem.*, **253**:7307-7313.

Regan, L.J., Sah, D.W.Y. and Bean, B.P., (1991) Ca^{2+} channels in rat central and peripheral neurons: high-threshold current resistant to dihydropyridine blockers and ω-conotoxin, *Neuron*, **6**:269-280.

Regehr E.G. and Atluri P.P., (1995) Calcium transients in cerebellar granule cell presynaptic terminals, *Biophys. J.*, **68**:2156-2170.

Régnier-Vigouroux A. and Huttner W. B., (1993) Biogenesis of small synaptic vesicles and synaptic-like microvesicles, *Neurochem. Res.*, **18**:59-64.

Říčný J., Čoupek J. and Tuček S., (1989) Determination of acetylcholine and choline by flow injection with immobilized enzymes and fluorometric or luminometric detection, *Anal. Biochem.*, **176**:221-227.

Robichaud L.J., Wurster S. and Boxer P.A., (1994) The voltage-sensitive Ca^{2+} channel (VSCC) antagonists ω-Aga-IVA and ω-CTX-MVIIC inhibit spontaneous epileptiform discharges in the rat cortical wedge, *Brain Res.*, **643**:352-356.

Roe M.W., Lemasters J.J. and Herman B., (1990) Assessment of Fura-2 for measurements of cytosolic free calcium, *Cell Calcium*, **11**:63-73.

Romano-Silva M.A., Gomez M.V. and Brammer M.J., (1994) The use of gadolinium to investigate the relationship between Ca^{2+} influx and glutamate release in rat cerebrocortical synaptosomes, *Neurosci. Letters*, **178**:155-58.

Rosen D.R., Siddique T., Patterson D., Figlewicz D. A., Sapp P., Hentati A., Donaldson D., Goto J., O'Regan J.P., Deng H.-X., Zohra R., Krizus A., McKenna-Yasik D., Cayabyab A., Gaston S.M., Berger R., Tanzi R.E., Halperin J.J., Herzfeldt B., Van den Bergh R., Hung W.-Y., Bird T., Deng G., Mulder D.W., Symth C., Laing N.G., Soriano E., Pericak-Vance M.A., Haines J., Rouleau G.A., Gusella J.S., Horvitz H.R., Brown R.H. Jr., (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis, *Nature* **362**:59-62.

Rowley H.L., Martin K.F. and Marsden C.A., (1995) Determination of in vivo amino acid neurotransmitters by high-performance liquid chromatography with o-phthalaldehyde-sulphite derivatisation, *J. Neurosci. Meth*, 57 :93-99.

Rowland L.P., (1991) Ten central themes in a decade of ALS research, *Advances in Neurology*, **56**:3-23.

Scheer H.W., (1990) Interactions between the presynaptically active neurotoxins α-latrotoxin and ω-conotoxin GVIA: studies on calcium fluxes and binding parameters in rat and chicken synaptosomes, *Can. J. Physiol. Pharmacol.*, **68**:1049-1054.

Scott R.H., Pearson H.A. and Dolphin A.C., (1991) Aspects of vertebrate neuronal voltage-activated calcium currents and their regulation, *Prog. Neurobiology*, **36**:485-520.

Scriabine A., Schuurman T. and Traber J., (1989) Pharmacological basis for the use of nimodipine in central nervous system disorders, *FASEB J.*, **3**:1799-1806.

Seagar M.J., Martin-Moutot N., Leveque C., El Far O., David P., Marqueze B., Lang B., Newsom-Davis J., Hoshino T. and Takahashi M., (1993) A Lambert-Eaton myasthenic syndrome antigen associated with presynaptic calcium channels, *Biochem. Soc. Trans.*, **21**:423-428.

Sen A.P., Boksa P. and Quirion R., (1993) Brain calcium channel related dihydropyridine and phenylalkylamine binding sites in Alzheimer's Parkinson's and Huntington's diseases, *Brain Res.*, **611**:216-221.

Sher, E., Biancardi, E., Passafaro, M., and Clementi, F., (1991) Physiopathology of neuronal voltage-operated calcium channels, *FASEB J.*, **5**:2677-2683.

Sherrington C.S., (1906) *The Integrative Action of the Nervous System*, C. Scribner's Sons: New York.

Sihra T.S. and Nichols R.A., (1993) Mechanisms in the regulation of neurotransmitter release from brain terminals: current hypotheses, *Neurochem. Res.*, **18**:47-58.

Singer D., Biel M., Lotan I., Flockerzi V., Hofmann F. and Dascal N., (1991) The roles of the subunits in the function of the calcium channel, *Science*, **253**:1553-1557.

Sitges M., Chiu L.M. and de la Fuente J.R., (1990) The effect of verapamil on GABA and dopamine release does not involve voltage-sensitive calcium channels, *Brain Res.*, **534**:51-59.

Smith R.G., Alexianu M.E., Crawford G., Nyormoi O., Stefani E. and Appel S.H., (1994) Cytotoxicity of immunoglobulins from amyotrophic lateral sclerosis patients on a hybrid motoneuron cell line, *Proc. Natl. Acad. Sci. U.S.A.*, **91**:3393-3397.

Smith R.G. and Appel S.H., (1995) Molecular approaches to amyotrophic lateral sclerosis, *Ann. Rev. Med.*, **46**:133-145.

Smith R.G., Hamilton S., Hofmann F., Schneider T., Nastainczyk W., Birnbaumer L., Stefani E. and Appel S.H., (1992) Serum antibodies to L-type calcium channels in patients with amyotrophic lateral sclerosis, *N. Engl. J. Med.*, **327**:1721-1728.

Spence K.T., Plata-Salaman C.R. and ffrench-Mullen J.M.H., (1991) The neurosteroids pregnenolone and pregnenolone-sulfate but not progesterone, block Ca^{2+} currents in acutely isolated hippocampal CA1 neurons, *Life Sci.*, **49**:PL235-PL239.

Südhof T.C., De Camilli P., Niemann H. and Jahn R., (1993) Membrane fusion machinery: insights from synaptic proteins, *Cell*, **75**:1-4.

Sun Y.-D. and Benishin C.G., (1989) Metal cations as synaptosomal calcium blockers in studies with Fura-2, *Neurochem. Res.*, **14**:1061-1066.

Suszkiw, J.B., O'Leary, M.E., Murawsky, M.M. and Wang, T., (1986) Presynaptic calcium channels in rat cortical synaptosomes: fast-kinetics of phasic calcium influx, channel inactivation, and relationship to nitrendipine receptors, *J. Neurosci.*, **6**:1349-1357.

Suszkiw, J.B., Murawsky, M.M. and Fortner, R.C., (1987) Heterogeneity of presynaptic calcium channels revealed by species differences in the sensitivity of synaptosomal ^{45}Ca entry to ω-conotoxin, *Biochem. Biophys. Res. Commun.*, **145**:1283-1286.

Suszkiw, J.B., Murawsky, M.M. and Shi, M., (1989) Further characterization of phasic calcium influx in rat cerebrocortical synaptosomes: inferences regarding calcium channel type(s) in nerve endings, *J. Neurochem.*, **52**:1260-1269.

Sze P.Y. and Iqbal Z., (1994) Glucocorticoid action on depolarization-dependent calcium influx in brain synaptosomes, *Neuroendocrinology*, **59**:457-465.

Tapia R., Sitges M. and Morales E., (1985) Mechanism of the calcium-dependent stimulation of transmitter release by 4-aminopyridine in synaptosomes, *Brain Res.*, **361**:373-382.

Tareilus, E. and Breer, H., (1992) Rapid kinetics of depolarization-induced changes in intrasynaptosomal calcium concentrations, *Neurochem. Int.*, **20**:275-279.

Tareilus E., Schoch J., Adams M. and Breer H., (1993) Analysis of rapid calcium signals in synaptosomes, *Neurochem. Int.*, **23**:331-341.

Tareilus E., Schoch J. and Breer H., (1994a) Ca^{2+}-dependent inactivation of P-type calcium channels in nerve terminals, *J. Neurochem.*, **62**:2283-2291.

Tareilus E., Schoch J. and Breer H., (1994b) $GABA_B$-receptor-mediated inhibition of calcium signals in isolated nerve terminals, *Neurochem. Int.*, **24**:349-361.

Terrian D.M., (1995) Persistent enhancement of sustained calcium-dependent glutamate release by phorbol esters: requirement for localized calcium entry, *J. Neurochem.*, **64**:172-180.

Thomas A.P. and Delaville F. (1991) The use of fluorescent indicators for measurement of cytosolic-free calcium concentration in cell populations and single cells, In: *Cellular Calcium: A Practical Approach*. pp. 1-53. Eds J.G. McCormack and P.H. Cobbold. IRL Press: Washington DC.

Thomas M.M., Puligandla P.S. and Dunn S.M.J., (1994) Effects of calcium channel blockers on the kinetics of voltage-dependent changes in synaptosomal calcium concentrations, *Brain Res.*, **635**:9-17.

Thomas P. (1987) The design and implementation of a CCITT V.32 modem on a general purpose digital signal processing microprocessor, *IEEE - ICC*, **87**:414-417.

Tibbs G.R., Dolly O. and Nicholls D.G., (1989a) Dendrotoxin, 4-aminopyridine, and β-bungarotoxin act at common loci by by two distinct mechanisms to induce Ca^{2+}-dependent release of glutamate from guinea-pig cerebrocortical synaptosomes, *J. Neurochem.*, **52**:201-206.

Tibbs G.R., Barrie A.P., Van Mieghem F.J.E., McMahon H.T. and Nicholls D.G., (1989b) Repetitive action potentials in isolated nerve terminals in the presence of 4-aminopyridine: effects on cytosolic free Ca^{2+} and glutamate release, *J. Neurochem.*, **53**:1693-1699.

Triggle D.J., (1994) Molecular pharmacology of voltage-gated calcium channels, *Ann. NY Acad. Sci.*, **747**:267-281.

Triggle D.J. and Janis R.A. (1987) Calcium channel ligands, *Ann. Rev. Pharmacol. Toxicol.*, **27**:347-369.

Tsien, R.Y. (1988) Fluorescence measurement and photochemical manipulation of cytosolic free calcium, *Trends Neurosci.*, **11**:119-124.

Tsien, R.Y., Pozzan, T. and Rink, T.T., (1982) Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator, *J. Cell Biol.*, **94**:325-334.

Turner T.J. and Dunlap K., (1995) Prolonged time course of glutamate release from nerve terminals: relationship between stimulus duration and the secretory event, *J. Neurochem.*, **64**:2022-2033.

Turner, T.J., Adams, M.E. and Dunlap, K., (1992) Calcium channels coupled to glutamate release identified by ω-Aga-IVA, *Science*, **258**:310-313.

Turner T.J., Adams M.E. and Dunlap K., (1993) Multiple Ca^{2+} channel types coexist to regulate synaptosomal neurotransmitter release, *Proc. Natl. Acad. Sci. U.S.A.*, **90**:9518-9522.

Turner T.J., Lampe R.A. and Dunlap K., (1995) Characterization of presynaptic calcium channels with ω-conotoxin MVIIC and ω-grammotoxin SIA: role for a resistant calcium channel type in neurosecretion, *Molec. Pharmacol.*, **47**:348-353.

Turner T.J., Pearce L.B. and Goldin S.M., (1989) A superfusion system designed to measure release of radiolabeled neurotransmitters on a subsecond time scale, *Anal. Biochem.*, **178**:8-16.

Uchitel, O.D., Protti, D.A., Sanchez, V., Cherksey, B.D., Sugimori, M. and Llinás, R.R., (1992) P-type voltage-dependent calcium channel mediates presynaptic calcium influx and transmitter release in mammalian synapses, *Proc. Natl. Acad. Sci. U.S.A.*, **89**:3330-3333.

Uski T.K., Högestätt E.D. and Andersson K.E., (1994) Clinical pharmacology of calcium channels, In:*Ion Channels and Ion Pumps*. pp. 190-225. Eds. P.P.Foà & M.F.Walsh. Springer-Verlag New York, Inc.: New York.

Venema, V.J., Swiderek, K.M., Lee, T.D., Hathaway, G.M. and Adams, M.E., (1992) Antagonism of synaptosomal calcium channels by subtypes of ω-agatoxins, *J. Biol. Chem.*, **267**:2610-2615.

Verhage M., Ghijsen W.E.J.M. and Lopes da Silva F.H., (1994) Presynaptic plasticity: the regulation of Ca^{2+}-dependent transmitter release, *Prog. Neurobiology*, **42**:539-574.

Vincent A., Barrett-Jolley R., Shillito P., Hart I., Beeson D., MacLennan C., Nicolle M., Lang B., Roberts M., Willison H. and Newsom-Davis J., (1994) Involvement of cation channels in autoimmune disease, *Biochem. Soc. Trans.*, **22**:488-491.

Waggoner A.S., (1979) Dye indicators of membrane potential, *Ann. Rev. Biophys. Bioeng.*, **8**:47-68.

Wheeler D.B., Randall A. and Tsien R.W., (1994) Roles of N-type and Q-type Ca^{2+} channels in supporting hippocampal synaptic transmission, *Science*, **264**:107-111.

White E.J., and Bradford H.F., (1986) Enhancement of depolarization-induced synaptosomal calcium uptake and neurotransmitter release by Bay K8644, *Biochem. Pharmacol.*, **35**:2193-2197.

Whittaker V.P., (1984) The synaptosome, In: *Handbook of Neurochemistry, Vol.7, 2nd ed.* pp. 1-37. Ed. J. Lajtha. Plennum Press: New York.

Whittaker V.P., (1993) Thirty years of synaptosome research, *J. Neurocytology*, **22**:735-742.

Wu L-G. and Saggau P., (1995) Block of multiple presynaptic calcium channel types by ω-conotoxin MVIIC at hippocampal CA3 to CA1 synapses, *J. Neurophysiol.*, **73**:1965-1972.

Yates, S.L., Fluhler, E.N. and Lippiello, P.M., (1992) Advances in the use of the fluorescent probe Fura-2 for the estimation of intrasynaptosomal calcium, *J. Neurosci. Res.*, **32**:255-260.

Young J.Z., (1975) Sources of discovery in neurosciences, In: *The Neurosciences: Paths of Discovery*, pp. 15-46. Eds. F.G. Worden, J.P. Swazey and G. Adelman. Colonial Press Inc.:Cambridge MA.

Zhainazorov A.B., Annunziata P., Toneatto S., Cherubini E. and Nistri A., (1994) Serum fractions from amyotrophic lateral sclerosis patients depress voltage-activated Ca^{2+} currents of rat cerebellar granule cells in culture, *Neurosci. Letts.*, **172**:111-114.

Zhang J.-F, Randall A.D., Ellinor P.T., Horne W.A., Sather W.A., Tanabe T., Schwartz T.L. and Tsien R.W., (1993) Distinctive pharmacology and kinetics of cloned neuronal calcium channels and their possible counterparts in mammalian CNS neurons, *Neuropharmacology*, **32**:1075-1088.

Zimmermann H., (1990) Neurotransmitter release, *FEBS Letters*, **268**:394-399.